

PROTEOGLYCANS IN SKELETAL TISSUES

A study of molecular events in
developing joint structures

Ahnders Franzén



Diss. Rerum.
1984



Organization LUND UNIVERSITY Department of Physiological Chemistry Faculty of Odontology P.O. Box 750 S-220 07 Lund, Sweden	Document name DOCTORAL DISSERTATION	
	Date of issue	1984-10-12
	CODEN:	LUODD5/(ODKE-1001)/1-136/(1984)
	Sponsoring organization	
Author(s) Ahnders Franzén		

Title and subtitle

PROTEOGLYCANS IN SKELETAL TISSUES. A study of molecular events in developing joint structures.

Abstract

The two main events in endochondral ossification are the cartilage calcification and the subsequent ossification at the surface of calcified cartilage remnants. The present investigation of the proximal epiphysis in the femoral head of growing rats revealed that initiation of calcification was accompanied by a decreased tissue content of proteoglycans and an increased proportion of those proteoglycans left in the calcified tissue were not capable of interaction with hyaluronic acid. The metabolic background to these alterations was further studied in vivo using ³⁵S-sulphate labelling of proteoglycans. At the onset of calcification, the biosynthetic rate decreased and the elimination rate increased of the epiphyseal cartilage proteoglycans, while no such changes were observed for the articular cartilage from the same femoral head. Preferentially the labelled aggregating proteoglycans decreased, indicating that proteoglycan aggregates may have an important role in the regulation of the calcification process.

Articular cartilages resist calcification, and one factor that may be involved are the proteoglycans. The relative content of aggregating proteoglycans was found to be quite high and well comparable with that in the epiphysis prior to calcification. However, close to the osteochondral border, the cartilage was calcified and contained a larger proportion of nonaggregating proteoglycans.

To improve our knowledge about the ossification process, proteoglycans were isolated and characterized from the calcified bone matrix. They were almost exclusively of low molecular weight, in contrast to the high proportion of high molecular weight proteoglycans seen in calcified cartilage matrix. The main bone proteoglycan had a molecular weight of only 76 000, had large chondroitin sulphate side chains and contained a well defined protein core of apparent Mw 46 000.

In a separate study, a procedure for separation of proteoglycans by zonal rate centrifugation in sucrose gradients was developed.

Key words

Articular cartilage, bone proteoglycan, calcification, cartilage proteoglycan, proteoglycan aggregate, sucrose gradient centrifugation.

Classification system and/or index terms (if any)
Supplementary bibliographical information

Language English

ISSN and key title

ISSN 91-7222-767-2

Recipient's notes

Number of pages 136

Price

Security classification
Distribution by (name and address)

Ahnders Franzén, Department of Physiological Chemistry, P.O. Box 750, S-220 07 Lund, Sweden.

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

Ahnders Franzén

Date August 22, 1984

PROTEOGLYCANS IN SKELETAL TISSUES

A study of molecular events in developing joint structures

AKADEMISK AVHANDLING

som för vinnande av doktorsexamen
vid Odontologisk Fakultet kommer
att offentligen försvaras i
Aulan, Tandläkarhögskolan, Malmö,
fredagen den 12 oktober 1984 kl. 09.00

av

Ahnders Franzén

leg. tandläkare

From the Departments of Physiological Chemistry and
Stomatognathic Physiology, University of Lund, Sweden

PROTEOGLYCANS IN SKELETAL TISSUES

A study of molecular events in
developing joint structures

Ahnders Franzén

Lund 1984

ISBN 91-7222-767-2

Printed in Sweden
Infotryck, Malmö 1984

To Monica, Henrik and Malin

CONTENTS

LIST OF PAPERS	5
ABBREVIATIONS	6
INTRODUCTION	7
Morphogenesis of skeletal tissues	7
Molecular constituents of cartilage and bone	10
BACKGROUND	13
Cartilage proteoglycans	13
Cartilage proteoglycans and calcification	17
Bone proteoglycans	19
PRESENT INVESTIGATION	21
Proteoglycans and calcification of cartilage in the femoral head epiphysis of the immature rat.	21
In vivo metabolism of ³⁵ S-sulphate labelled proteoglycans in the epiphyseal, articular and nasal cartilages of growing rats. ...	23
Variations in the composition of bovine hip articular cartilage with distance from the articular joint surface.	25
Extraction and purification of proteoglycans from mature bovine bone.	27
Characterization of proteoglycans from the calcified matrix of bovine bone.	29
Zonal rate centrifugation of proteoglycans in sucrose gradients. ..	30
CONCLUDING REMARKS	32
ACKNOWLEDGEMENTS	34
REFERENCES	36
APPENDIX: PAPERS I-VI	41

This thesis is based on the following papers which are referred to by their Roman numerals.

- I. Proteoglycans and calcification of cartilage in the femoral head epiphysis of the immature rat.
Franzén, A., Heinegård, D., Reiland, S. and Olsson, S.-E.
J. of Bone and Joint Surg. Vol. 64-A, No 4, 558-566, 1982
- II. In vivo metabolism of ^{35}S -sulphate labelled proteoglycans in epiphyseal, articular and nasal cartilages of growing rats.
Franzén, A. and Heinegård, D.
In manuscript
- III. Variations in the composition of bovine hip articular cartilage with distance from the articular surface.
Franzén, A., Inerot, S., Hejderup, S.-O. and Heinegård, D.
Biochem. J. 195, 535-543, 1981
- IV. Extraction and purification of proteoglycans from mature bovine bone
Franzén, A. and Heinegård, D.
Biochem. J. in press
- V. Characterization of proteoglycans from the calcified matrix of bovine bone.
Franzén, A. and Heinegård, D.
Biochem. J. in press
- VI. Zonal rate centrifugation of proteoglycans in sucrose gradients.
Franzén, A., Björnsson, S. and Heinegård, D.
Anal. Biochem. 120, 38-45, 1982

ABBREVIATIONS

Dwt - dry weight

EDTA - ethylenediaminetetraacetate

FITC - fluorescein isothiocyanate

S - sedimentation coefficient, expressed in Svedberg units

SDS - sodium dodecyl sulphate

Morphogenesis of skeletal tissues

Major functions of the skeleton are to provide support and protection for the internal organs and to create a structure allowing mobility. The latter function is obtained by the presence of joints in the rigid skeleton, where the different parts can move one against the other. The design of these joints, however, is critical since a requirement is smooth movement during a large number of years. In the adult, the joint (outlined in figure 1) is formed by two bones, where the ends are covered with hyaline articular cartilage. The latter, then forms the contact, articulating surface between the two bones and also forms the interface to the synovial fluid, which provides nutrients for the cartilage. The whole joint is enclosed by the joint capsule.

Although bone and cartilage are extremely different tissues both with respect to structure, composition and function, they develop from the same cartilaginous structure during the skeletogenesis.

For example like many other tubular bones in the body the femur is first formed as a precursor of hyaline cartilage containing two structurally different regions, i.e. the two ends or epiphyses interposed by the shaft or diaphysis (Fig. 2a). Prenatally, the diaphyseal cartilage is replaced by bone (primary ossification), while the majority of the cartilaginous epiphyses are ossified (secondary ossification) only after birth (schematically shown in Fig. 2a-d). The mechanism whereby the cartilaginous matrix is replaced by bone is known as endochondral ossification (for reviews see Bloom and Fawcett, 1975; Ogden 1979; Ham and Cormack, 1979). This process can be exemplified with the development of the femoral head. At the onset of this secondary ossification the cells in the cartilage, (i.e. the chondrocytes) hypertrophy centrally in the femoral head epiphysis. Amorphous calcium phosphate is then deposited in the matrix between the chondrocytes and is subsequently transformed to hydroxyapatite crystals (Fig. 3b). The calcified, still cartilaginous matrix is eroded and invaded by capillaries with a concomitantly ingrowth of undifferentiated mesenchymal cells. Probably influenced by environmental factors, these cells are transformed into osteoblasts, which produce unmineralized bone matrix (i.e. osteoid) on remnants of calcified cartilage (Fig. 3c). The osteoid is mineralized after some ten days, at a time when hydroxyapatite crystals constitute about 70 % of the bone matrix. After the secondary ossification



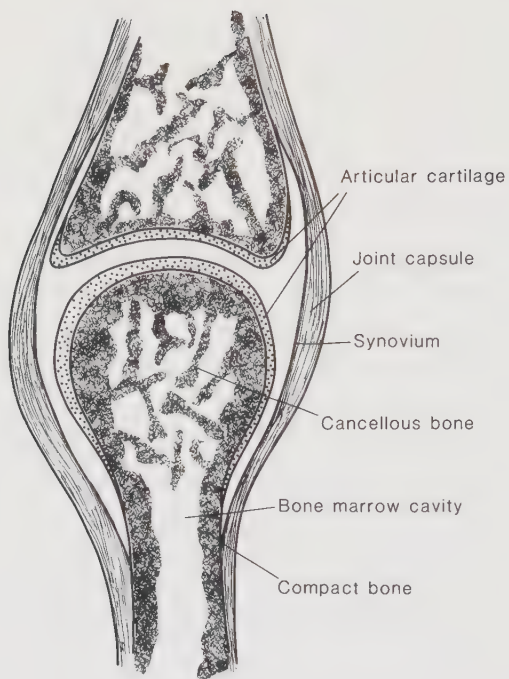


FIGURE 1: Schematic illustration of a diarthrodial joint.

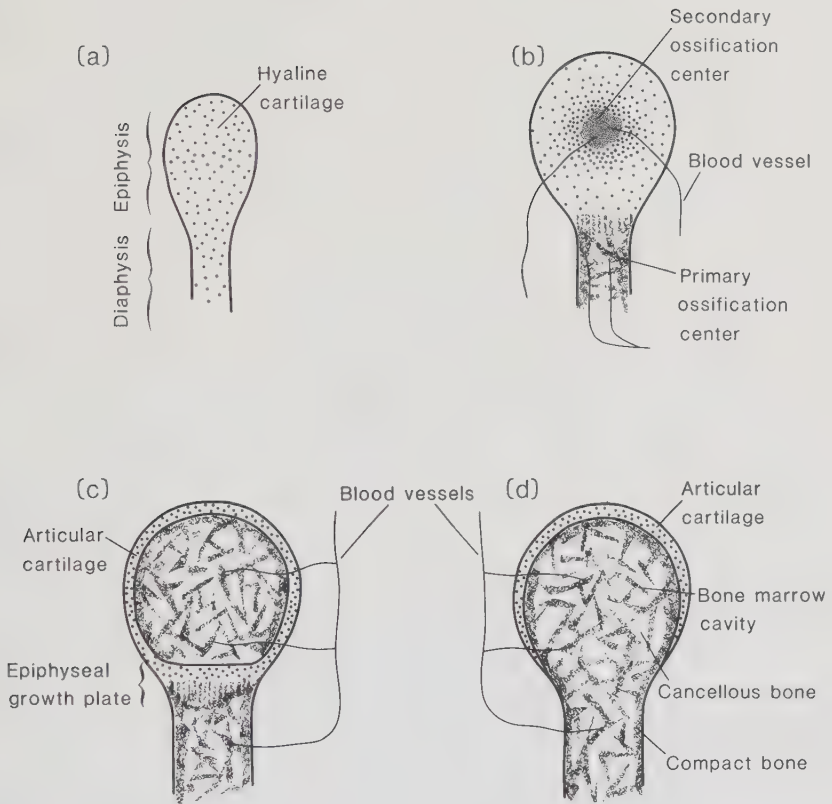


FIGURE 2 (a-d): Schematic illustration of growth and development of a femoral head.

is complete two cartilaginous structures remain - the epiphyseal growth plate and the articular cartilage (Fig. 2c). Those structures are extremely important for the further growth and function of the skeleton. Within the epiphyseal growth plate, the longitudinal growth of the extremities is maintained by a continuous, directed growth of the cartilaginous matrix caused by proliferating chondrocytes and an increased matrix production. The cartilaginous matrix in one end of the epiphyseal growth plate is continuously calcified and ossified. The longitudinal growth of the bones comes to an end, in human approximately at puberty (Slavkin, 1979), when the growth of the epiphyseal cartilage ceases and the whole structure is ossified. The articular cartilage, in contrast, persists throughout life. At present, it is unknown what factors allow the articular cartilage to resist calcification and subsequent ossification.

Molecular constituents of cartilage and bone

Cartilage and bone, like many other connective tissues, have very prominent extracellular matrices, that are maintained by the relatively few cells in the tissues. The two major constituents of cartilage, synthesized by its cells, the chondrocytes, are collagens and proteoglycans although the tissue also contains a number of other proteins in low concentration, i.e. lysozyme (Kuettnet et al., 1971), linkproteins, 148 kDa protein, chondronectin, fibronectin and minor collagens (for review see Paulsson and Heinegård, 1984). The predominant collagen is referred to as type II collagen (Miller and Matukas, 1969) and is unique to cartilage and the vitreous body. This macromolecule is a typical interstitial fiber forming collagen, which provides the tissue with a network of fibers; a prerequisite for its tensile properties and essential for the maintenance of the tissue volume. In this collagen network, the highly polyanionic proteoglycans (for review see Heinegård and Paulsson, 1984), create a high osmotic pressure with the result that water is retained and the tissue volume expands, but only to the limits imposed by the collagen fibers (Kempson et al., 1970). At all times, however, there is a high swelling pressure of connective tissues.

The predominant constituent of bone is the mineral, i.e. hydroxyapatite, which represents about 70 % of the dry weight. The major organic constituent is the type I, interstitial collagen, which forms some 90 % of the organic mass (Miller and Martin, 1968). Among other components, notably there are several polyanionic macromolecules like phosphoproteins (Schutt-

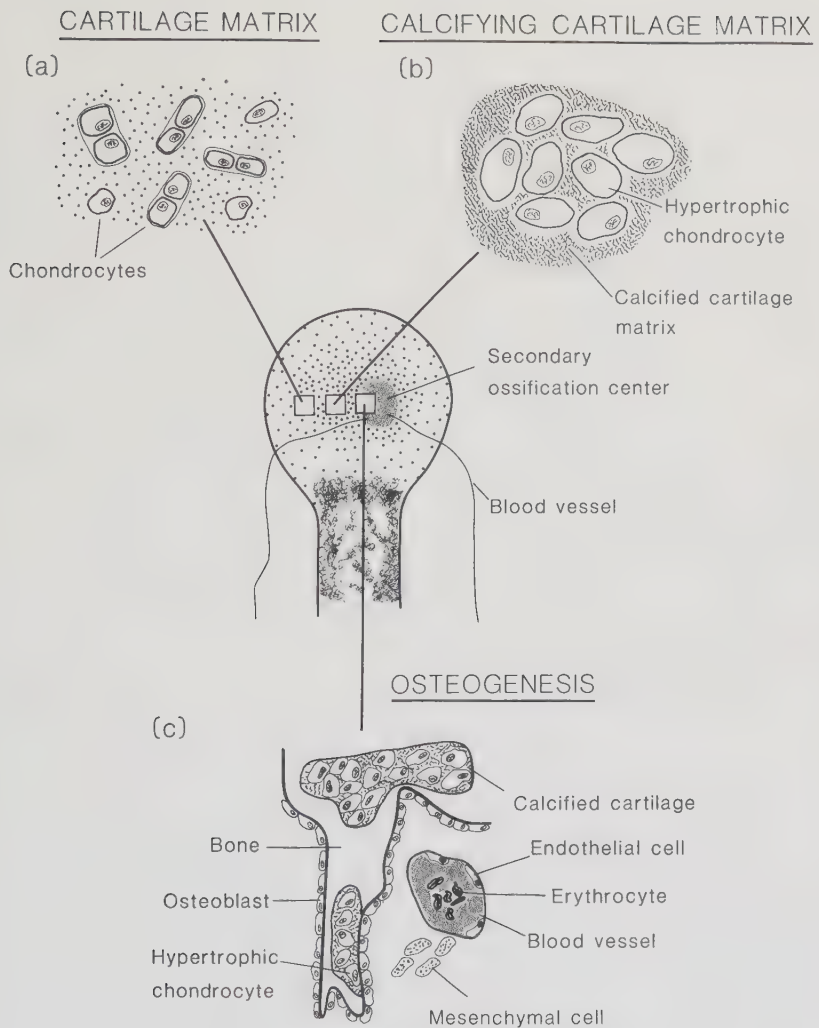


FIGURE 3 (a-c): Outline of events in endochondral ossification.

leworth and Veis, 1972; Spector and Glimcher, 1972), proteoglycans (Herring, 1968; Fischer et al., 1983a), sialoproteins (Herring and Kent, 1963; Fischer et al., 1983b) osteocalcin (Hauschka et al., 1975; Price et al., 1976) and osteonectin (Termine et al., 1981). Fibronectin (Weiss and Reddi, 1981) and several plasma proteins (Ashton et al., 1976) have also been identified in the bone.

The role of the various macromolecules and cells in the processes leading to bone formation and also in the maintenance of normal cartilage and bone structure and function have been much discussed. Although so called matrix vesicles, that represents structures that bud off from the chondrocyte, have been proposed to be involved in the initial stages of calcium deposition (Cecil and Anderson, 1978) more recent data suggests other roles (for ref. see Reinholt, 1983). This presentation will focus on the proteoglycans, molecules that because of their high charge density can provide elasticity to a cartilage and aid in distributing forces occurring under excessive loading. These polyanionic molecules can also be an important factor in the regulation of calcium phosphate deposition as well as in the transformations of amorphous calcium phosphate to crystalline hydroxyapatite (Anderson, 1980; Boskey, 1981). Although little is known of the exact function of the proteoglycans more insight can be gained from studies of the changes they undergo in development and differentiation during animal growth. Some insight has already been gained from studies of proteoglycans in defects of skeletogenesis, i.e. various forms of short stature and dwarfism. In many of these cases a lowered matrix concentration of proteoglycans or the presence of proteoglycans with altered properties have been noted (Orkin et al., 1976; Kimata et al., 1981), while the content of type II collagen appears normal. Furthermore, similar skeletal disturbances have been induced in rabbits by the injection of the protease papain (Hult, 1958). It is known that papain will depolymerize the proteoglycans in the cartilage, while the collagen molecules are more resistant to depolymerization.

It appears that the proteoglycans have an important function in maintaining cartilage function. Available data also indicate that they are involved in regulating at least the cartilage calcification during the endochondral ossification. A more detailed description of the structure of these molecules is therefore given below.

Cartilage proteoglycans

Over the years studies on the structure and properties of proteoglycans have been centered on those from cartilage. This has mainly been due to the relative ease of preparing the molecules from this tissue, compared with other tissues where the relative contents of proteoglycans may be from ten to a thousand times lower. Still, a central problem has been to prepare intact, pure molecules in good yields. Major breakthroughs were the introduction (Franek and Dunstone, 1967) of cesium chloride gradient centrifugation to separate proteoglycans from proteins and the combination of this technique with the extraction of proteoglycans from the tissue with 4M guanidine-HCl introduced by Sajdera and Hascall (1969). This procedure improved the yield of cartilage proteoglycans several-fold to 85-90% and has since been shown to be applicable also to other types of tissues. The use of the novel techniques during the 1970-ies has allowed the preparation of large quantities of pure proteoglycans, which has been used in studies to further the knowledge on their structure.

The cartilage proteoglycan is an extremely large molecule, having an average molecular weight of $2-3 \times 10^6$ dalton (Hascall and Sajdera, 1970). The molecule contains a central protein core, accounting for about one tenth of the total molecular weight (Hascall and Riolo, 1972). The core is substituted with about 80-100 covalently bound chondroitin sulphate chains, about 50 keratan sulphate chains, some 50 O-glycosidically linked oligosaccharides and 5-15 N-glycosidically linked oligosaccharides (Heinegård et al., 1984), schematically shown in Fig. 4. The number of different substituents, then, is very large. Three regions of the proteoglycan molecule that differs with respect to substituents and composition of the protein have been identified. At one end of the core the majority of the chondroitin sulphate chains are bound in the chondroitin sulphate attachment region. A middle region of the molecule that represents some 10% of the protein core contains many of the keratan sulphate chains in the keratan sulphate rich region (Heinegård and Axelsson, 1977). The remaining part of the molecule, the hyaluronic acid binding region, forming the other end, contains about 1/4 of the protein (Heinegård and Hascall, 1974a). It has a globular structure (Perkins et al., 1981) and does not contain covalently attached glycosaminoglycan chains (Heinegård and

Hascall, 1974a). It contains, however, all of the N-glycosidically linked oligosaccharides in the proteoglycan. The O-glycosidically linked oligosaccharides are distributed over both the chondroitin sulphate rich region and the keratan sulphate rich region. This type of oligosaccharides is of the mucin type being linked to serine or threonine and having a structure similar to that of the keratan sulphate linkage to protein, i.e. via N-acetylgalactosamine (DeLuca et al., 1980; Lohmander et al., 1980). The N-linked oligosaccharides are of the complex type linked to asparagine and being either biantennary or triantennary (Nilsson et al., 1982). The chondroitin sulphate chains are linked via a characteristic linkage region by xylose to serine in the protein core (for review see Rodén, 1980). The protein content of proteoglycans may vary, such that those isolated from bovine nasal and tracheal cartilages contain about 10 % protein, while those from articular cartilage contain as much as 25-30% (Muir, 1980). Typically, the prominent amino acids in the cartilage proteoglycans are serine, glycine and glutamic acid (for ref. see Heinegård and Paulsson, 1984).

The cartilage proteoglycans can, in a very specific manner interact with a very large glycosaminoglycan present in cartilage matrix, i.e. hyaluronic acid. Several proteoglycan molecules bind via their hyaluronic acid binding region to each hyaluronic acid molecule, forming a very large proteoglycan aggregate (Hardingham and Muir, 1972; Hascall and Heinegård, 1974b). One function of such an aggregate may be to form a molecular organization for the distribution of negative charges in the tissue. Although the interaction between hyaluronate and proteoglycan is strong having a dissociation constant of about 10^{-8} (Christner et al. 1978, Nieduszyński et al. 1980), it is stabilized by a distinct protein, the link protein, which can specifically interact both with the hyaluronate (Tengblad, 1981) and with the proteoglycan monomer (Hardingham, 1979; Heinegård and Hascall, 1979a; Franzén et al., 1981).

Available data show that cartilage contains link stabilized proteoglycan aggregates (Hascall and Heinegård, 1974a) and that such aggregates can be isolated from epiphyseal (Howell et al., 1968) and tracheal (Paulsson and Heinegård, 1979) cartilages as well as from rat chondrosarcoma (Faltz et al., 1979). The aggregates are formed extracellularly (Kimura et al., 1979; Björnsson and Heinegård, 1981) but in rather close context with the secretion of the components from the cells.

With improved techniques available to the biochemist it has become possible to identify subpopulations of proteoglycans having quite different cha-

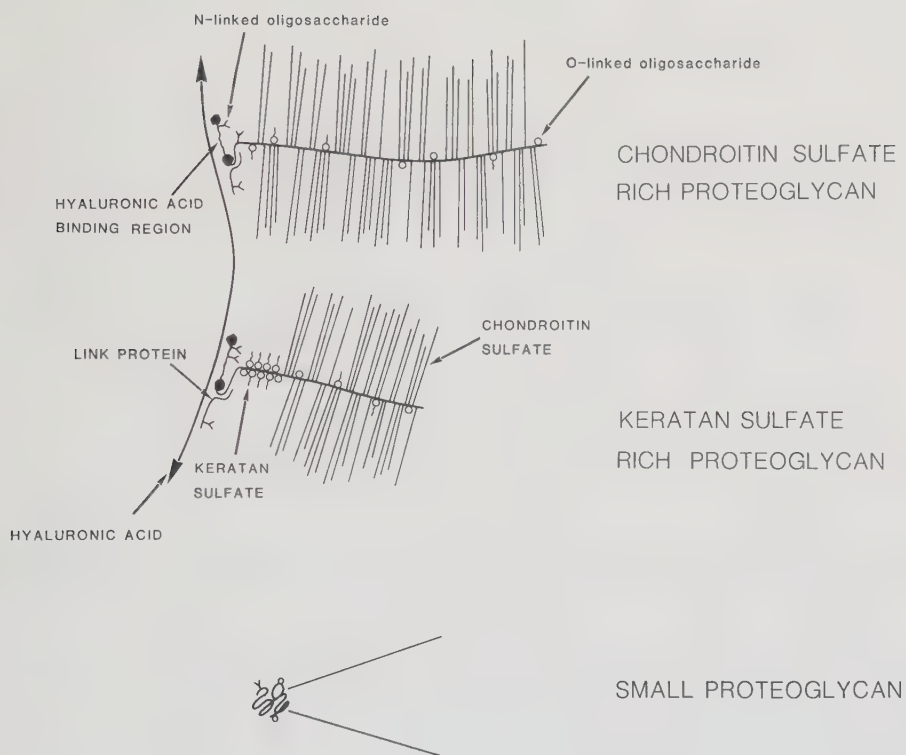


FIGURE 4: Tentative models of cartilage proteoglycans.

racter. Cartilage can be used as an example since it appears to contain molecules similar in character to those subpopulations present in other tissues. Exceptions are the heparan sulphate proteoglycans that appear not to be present in either cartilage or bone and a keratan sulphate proteoglycan unique for cornea (for ref. see Heinegård and Paulsson, 1984). Nevertheless, cartilage contains at least four apparently dissimilar proteoglycans, three of them outlined in figure 4. There are two populations of high molecular weight, aggregating proteoglycans (Heinegård et al., 1981a), one having a high relative content of chondroitin sulphate (CS), the CS-rich proteoglycan, and the other having a high relative of keratan sulphate (KS), the KS-rich proteoglycans. It should be stressed, however, that both proteoglycans contain all types of side chain substituents, but in different proportions. They differ in size, 3.5×10^6 and 1.3×10^6 , respectively, and with respect to their protein core, as shown by amino acid composition and by peptide patterns (Heinegård, personal communication). It is presently not known with certainty if they have a product-precursor relationship. Both contain a hyaluronic acid binding region of similar structure and interact with the same specificity with hyaluronate.

A third population is the high molecular weight nonaggregating proteoglycans with a high protein content (Heinegård and Hascall, 1979b). Its amino acid composition, however, is typical for the portion of the aggregating proteoglycans containing the chondroitin sulphate chains. It appears to represent a distinct entity, since any fragments of the aggregating proteoglycans having a similar amino acid composition would most likely also have a low protein content. This fraction, though, may also contain variable amounts of fragments of the aggregating proteoglycans, resulting from normal or increased catabolism.

The fourth subpopulation represents a very different class of small, nonaggregating proteoglycans (Heinegård et al., 1981b). Its molecular weight is 76,000 and the molecule has a 3-4 times higher protein content compared with the large proteoglycans, i.e. about 30% yielding a core of about 30,000 dalton. The amino acid composition is unique, with very high contents of leucine, aspartic acid/asparagine and glutamic acid/glutamine. The chondroitin sulphate side chains are large, i.e. Mw about 35,000 compared with a Mw of less than 20,000 for the large proteoglycans. Interestingly, similar small proteoglycans, with the large side chains being either chondroitin sulphate or dermatan sulphate, have been identified in many tissues like aorta (Gardell, personal communication), cornea (Axelsson and Heinegård, 1980), cervix (Uldbjerg et al., 1983), tendon (Vogel

and Heinegård, to be published) and sclera (Cöster and Fransson, 1981). Indeed many tissues also appear to contain two types of aggregating proteoglycans closely similar to those found in cartilage, but devoid of keratan sulphate chains. In nasal cartilage, the two aggregating proteoglycans together represent 80-85% of the total, while the figure for articular cartilage is lower, i.e. about 50%. The large nonaggregating proteoglycans represent 10% of the total, while the small proteoglycan only represent 2-3%. The latter, however, accounts for almost half the number of proteoglycan molecules. In other tissues, the small proteoglycans are predominating and represents as much as 90% of the total proteoglycan mass. Except for the fact that the proteoglycan aggregates are assembled extracellularly, very little is known about their metabolism in the matrix. It is assumed that they are catabolized primarily by proteolytic degradation, the fragments becoming free to diffuse to the cells or out of the cartilage and further processed. The role of the cartilage proteoglycans in various processes was mentioned above. The currently available data on their role in matrix calcification deserves additional discussion.

Cartilage proteoglycans and calcification

The potential involvement of proteoglycans in the regulation of processes like cartilage calcification briefly mentioned above, has been the subject of many studies (for review see Buchwalter, 1983). Proteoglycan aggregates appear to be an important inhibitor of calcification, by preventing nucleation and transformation of amorphous calcium phosphate to hydroxyapatite. The effects of proteoglycans in systems for in vitro calcification as well as the changing structure of proteoglycans during calcification of the tissue has been studied. For example, the effects of proteoglycan aggregates, proteoglycan monomers and fragments thereof upon the formation of hydroxyapatite crystals were tested in vitro using precipitating calcium phosphate solutions (Cuervo et al., 1973; Howell and Pita, 1976; Blumenthal et al., 1980). The proteoglycan aggregates were more potent in preventing formation of hydroxyapatite crystals than the proteoglycan monomers. When the proteoglycan aggregates were degraded by proteinases the inhibition was notably reduced. There are, however, many conflicting results as to whether or not proteoglycan aggregates or monomers decrease in the calcifying cartilage matrix. Thus, reports describe a net loss of proteoglycans from calcifying cartilages (Hirschman and Dziewiatkowski,

1966; Thyberg, 1974; Lohmander and Hjerpe, 1975) or an unchanged content of proteoglycans (Weatherell and Weidman, 1963; Lindenbaum and Kuettner, 1967; Mitchell et al., 1982). Recently, Poole et al. (1982) using immunolocalization of proteoglycans and link proteins in the epiphyseal growth plate suggested that these molecules were the same in calcified compared with not calcified cartilage. Studies of the detailed structure of proteoglycans isolated from calcifying cartilage, however, suggested that the proportion of nonaggregating proteoglycans or fragments thereof was higher in calcifying cartilage compared with that noncalcified (Lohmander and Hjerpe, 1975; Vittur et al., 1977; Pita et al., 1979).

Few studies of the macromolecules in the substructures of the normal typical epiphysis have been conducted, mainly because of problems in preparing defined tissue fractions. As an alternative, the normal calcification has been interfered with, as in experimental rickets. The widening of the epiphysis that ensures facilitates micromanipulations. Studying such epiphyses from rachitic rats, Pita et al., (1979) could withdraw extracellular fluid from the hypertrophic cartilage and show a high content of very large proteoglycan aggregates. Upon healing of the rickets the tissue contents of these superaggregates gradually decreased. In other studies endochondral bone formation have been induced by subcutaneous implantation of demineralized bone powder. First a cartilage nodule is formed, which is calcified and develops then into an ossicle (for ref see Reddi, 1981). Although the tissue content of proteoglycans was lower in the calcified cartilage, it was not possible to demonstrate any alterations of the structure of the remaining proteoglycans (Reddi et al., 1978). Taken together there is an abundance of reports suggesting a decreasing content of proteoglycans, primarily aggregates, upon calcification. The mechanism is not known: although it has been suggested that proteinases may be involved, supported by the higher content of proteinase activity in calcified compared with that in not calcified cartilage (Granda and Posner, 1971; Tebor et al., 1982). Other studies, however, have failed to demonstrate increased proteinase activity in fluid withdrawn from the hypertrophic zone of healing rickets, i.e. where contents of proteoglycans are decreasing (Howell and Pita, 1976). Another factor that has been implemented is lysozyme, found in relatively high quantities in epiphyseal cartilage. This basic protein has been shown to dissociate the very large proteoglycan aggregates in vitro (Kuettner et al., 1974; Pita et al., 1975; Tang et al., 1981). Yet another possibility is an increased synthesis of a proteoglycan not capable of forming aggregates.

What could be the effect of the proteoglycans, in particular the aggregates? It has been suggested that particularly the aggregates may interfere with nucleation or may sequester newly formed hydroxyapatite crystals from the site of calcification. The extremely polyanionic proteoglycans may also be involved in determining free concentrations of the calcium ions, important for the salt precipitation. Furthermore, they may bind to the newly formed amorphous calcium phosphate thereby preventing its transformation to the stable crystalline hydroxyapatite. Much work is, however, still required for a better understanding of the process (for ref. see Buckwalter, 1983).

Bone proteoglycans

Endochondral ossification is characterized by several steps involving differentiation of cells and changes in matrix composition. One step is the calcification discussed above, and another is the ossification, i.e. cells differentiate to osteoblasts capable of forming the bone matrix, which probably contains a quite different set of molecules compared with cartilage. This is well known for the collagens. One question is whether the proteoglycans that form such a major component in cartilage are the same in bone or whether other types of proteoglycans predominate. Unfortunately our knowledge about the structure and composition of bone matrix proteoglycans are sparse and even confusing. There are several reasons, the first being that there are problems involved in extraction, i.e. the liberation of the proteoglycans from their association with the bone mineral. The extraction procedure most often used in the past involved demineralization of the bone (i.e. solubilization of the hydroxylapatite crystals) with EDTA during several days or sometimes even during several weeks (Herring, 1968). Better control, by including protease inhibitors was, however, obtained when powdered bone was demineralized with EDTA in the presence of 4 M guanidine hydrochloride (GuHCl) (Engfeldt and Hjerpe, 1976; Reddi et al., 1978). Secondly, bone is a very heterogeneous tissue compared with cartilage. It is composed of several non-mineralized tissues like blood vessels, bone marrow and fibrous tissues in addition to the mineralized bone matrix (Bloom and Fawcett, 1975). When bone is demineralized with EDTA or with EDTA in the presence of 4 M GuHCl, several macromolecules including the proteoglycans are extracted from the non-mineralized as well as from the mineralized tissue components in the bone. Any proteoglycan unique for the mineralized matrix is therefore contaminated with pro-

teoglycans from the other structures present.

Recently, proteoglycans have been isolated from immature bovine rib bone using a sequential extraction procedure (Fischer et al., 1983a), a technique almost identical to that developed and used in the present investigation (paper IV). The strategy chosen was primarily to solubilize molecules not associated with mineral by extraction with 4 M GuHCl and proteinase inhibitors, omitting EDTA. The residue was subsequently extracted with extraction solvent also containing EDTA, which dissolved the bone mineral allowing those molecules associated with the mineral to be solubilized. A small proteoglycan containing side chains of chondroitin sulphate was identified in and purified from the subsequent extract with GuHCl-EDTA. Its protein content was about 20 % (of dwt) and the predominant amino acids were leucine, aspartic acid/asparagine and glutamic acid/glutamine (Fischer et al., 1983a).

PRESENT INVESTIGATION

(The roman numerals within brackets refer to papers in the thesis)

The aim of the present study has been to improve the understanding of the regulation of endochondral ossification by studies of the molecular changes of the proteoglycans during the process. Major focus has been put on the calcification and the ossification, both processes probably involving drastic changes of proteoglycans and important for the properties of the bone formed. For establishing those changes in proteoglycan biochemistry that occur during calcification (I, II), the femoral head epiphysis of growing rats has been used, mainly because processes like proliferation, hypertrophy, and calcification occur at different times facilitating preparation of tissue samples in which mainly one particular process is under way. The specificity of the process for the epiphyseal cartilage has been determined by comparing samples from epiphyseal and articular cartilage, respectively. The final transformation to bone has been studied by way of establishing the different character of the bone proteoglycans (IV, V), compared with the character of the proteoglycans in the calcified epiphyseal cartilage and in the articular cartilage (III). A sensitive zonal rate centrifugation procedure for monitoring molecular dimensions of the proteoglycans at various stages of the processes has been developed (IV).

Proteoglycans and calcification of cartilage in the femoral head epiphysis of the immature rat (I)

The secondary ossification (i.e. cartilage matrix calcification and bone formation) in the rat femoral head differs somewhat in timing from that in human and most animals. Calcification and subsequent ossification occur separately with respect to time and initial location. The calcification of the rat femoral head begins centrally at three weeks after birth (day 21). The process then continues for another three weeks until the whole epiphysis is calcified only sparing the articular cartilage in the periphery (Koshino, 1975). The first morphological sign of osteogenesis is then seen on the surface of calcified cartilage remnants, laterally in the femoral head at day 45-50 (Koshino and Olsson, 1975). The long time between the two processes make this epiphysis suitable for biochemical characterization of events in endochondral ossification.

The present study was aimed at characterizing the cartilage proteoglycans

in the growing rat femoral head to disclose any changes at the time of calcification. To give an overall view, the total amounts of proteoglycans and collagen, the two major constituents of cartilage, were quantitated in the rat femoral head at different ages before and during calcification of the epiphysis. The wet weight of the femoral head and its content of collagen increased in an almost linear fashion with age, indicating the growth of the animal with age. Also the tissue content of proteoglycans increased in a linear fashion and parallel to that of collagen until the age of 20 days, one day prior to the onset of calcification. Subsequently the content of proteoglycans increased more slowly compared with collagen. The relatively lower tissue content of proteoglycans with advancing calcification indicates that these molecules might be involved in the regulation of the calcification process. A decreasing tissue content of proteoglycans during cartilage calcification has similarly been observed by others (Hirschman and Dziewiatkowski, 1966; Lohmander and Hjerpe, 1975). To obtain further support and demonstrate any structural changes, proteoglycans were extracted from femoral heads of rats of different ages. The proteoglycans were then purified by cesium chloride density gradient centrifugation and characterized. Their composition changed with increasing age of the animal and with advancing cartilage calcification. Thus contents of keratan sulphate increased compared with contents of chondroitin sulphate and of protein. Furthermore at onset and with ongoing calcification the proteoglycan monomers became somewhat smaller, compared with those prepared from the femoral head prior to calcification. The changes may indicate either the synthesis of an altered proteoglycan or fragmentation of a proteoglycan already present in the matrix and subsequent loss of some of the fragments. Assuming a fragmentation, the average size of the proteoglycan monomers could be reduced in many different ways, i.e. due to shorter glycosaminoglycan side chains, a reduced number of chondroitin sulphate side chains along the proteoglycan core protein or/and a decreased length of the core protein due to protease cleavage. Further information was therefore gained by studying the distribution and the size of the side chains, which is done by determining size distributions of chondroitin sulphate peptides produced by trypsin digestion (Heinegård and Hascall, 1974b) and by determining the size of the chains by papain digestion (Wasteson, 1971), respectively. Both the distribution of the glycosaminoglycan chains along the core protein and the average size of the glycosaminoglycan chains was constant over all ages. Therefore, it is most likely that the proteoglycan monomers became smaller due to the core protein becoming

smaller and therefore containing fewer side chains. Furthermore, the proportion of the proteoglycans capable of forming aggregates with added hyaluronic acid was reduced at the onset of the calcification in a good agreement with results obtained by Lohmander and Hjerpe (1975). Thus, both at day 20 and at day 40 of age the proportion of aggregating proteoglycans was lower compared with that in the non calcified matrix at younger age. At day 25, however, the proportion of aggregating proteoglycans was somewhat higher and the proteoglycan monomers were slightly larger than observed at day 20 and day 40. It should be born in mind that the proteoglycans to be characterized were prepared from the whole cartilaginous femoral head and changes occuring in the articular cartilage may disguise those changes occuring in the epiphyseal cartilage. The alterations observed, however, indicate that the proteoglycans participate in the calcification process and that they are removed at the time of calcification although their function can not be deduced from the present study.

In vivo metabolism of ^{35}S -sulphate labelled proteoglycans in the epiphyseal, articular and nasal cartilages of growing rats (II)

As discussed the changes in proteoglycan quantity and structure may either result from an increased catabolism or from a depressed synthesis. To shed some light on this question studies of proteoglycan turnover in the living animal was undertaken using radio-tracer techniques. To allow monitoring of events specific for the epiphysis, a procedure was developed to separate this part of the femoral head from the articular cartilage. The turnover of the proteoglycans in these two tissue components could therefore be compared and was furthermore compared also to that in a cartilage from an entirely different location, i.e. the nasal cartilage.

The tracer chosen was ^{35}S -sulphate that specifically will label proteoglycans after intraperitoneal injection (Boström, 1952). Two types of protocols were used. To study the biosynthesis, isotope was injected at various ages of the animal, only female rats being used. These rats were killed 24 hours after the injection. Labelled proteoglycans were isolated from the different tissue portions and the total amount of radioactivity in the proteoglycans were taken as a measure of the biosynthetic rate over 24 hours. In the alternative protocol used to study proteoglycan catabolism, ^{35}S -sulphate was injected in young female rats, i.e. at day 13. The animals were then killed after variuos time intervals. It was assumed that proteoglycans were only labelled during the 12 hour pulse used and that no

or negligible amounts of isotope was reutilized. Consequently, the amount of ^{35}S -proteoglycans remaining in the different tissue portions at various ages would give an indication of their elimination rate and any changes of their structure would give information on the nature of the processes involved.

The data interestingly show that both biosynthesis and catabolism of the proteoglycans are altered. The biosynthesis decreases at the onset of the calcification process, most markedly in the epiphyseal cartilage and less so in the articular cartilage. Reddi et al. (1978) similarly showed that the biosynthetic rate of proteoglycans in calcifying cartilage is lower compared with that in not calcifying cartilage. Thus one explanation for the decreasing proteoglycan concentration is reduced biosynthesis of the molecules, impairing the replacement of those molecules catabolized.

Furthermore, the catabolism was much increased. About 2-3 days prior to calcification of the epiphysis the ^{35}S -sulphate labelled proteoglycans in the epiphyseal cartilage were eliminated from the tissue with a much higher rate, than observed in the articular and nasal cartilages. However, with ongoing calcification of the epiphysis also the labelled articular cartilage proteoglycans were eliminated at an increased rate, but lower than that in the epiphyseal cartilage, while the elimination of proteoglycans from the nasal cartilage was very low. Also the structure of those proteoglycans remaining in the cartilage changed with age and time after the isotope injection most markedly in the epiphyseal cartilage. With time after the isotope injection the labelled proteoglycans became smaller and a larger proportion could not form aggregates with hyaluronate. Those from the articular cartilage showed only minor changes.

The data show that the catabolism of proteoglycans in the epiphyseal cartilage probably are eliminated by proteolysis, the fragments free to diffuse out of the tissue. This result fits well with observations (Granda and Posner, 1971; Tebor et al., 1982) that calcifying cartilage has a high content of proteinases, some of which have a high capacity to degrade proteoglycans (Sapolsky et al., 1981). It is possible that there is some preferential degradation of aggregating proteoglycans. Fellini et al. (1981), studying an in vitro system, obtained indications that proteoglycans are synthesized as components with a rather monodisperse protein core. The present study, then, indicates that the polydispersity of the bulk of cartilage proteoglycans may arise from progressive depolymerization by gradual proteolysis, supporting observations by Oegema et al. (1979) and McDevitt et al. (1981).

In conclusion, the decreased content of proteoglycans in calcifying cartilages is due to a combination of a decreased biosynthesis and an increased elimination rate of proteoglycans. The regulating mechanism for these alterations is not known. It is possible that a changing hormonal balance may induce the epiphyseal chondrocytes and to a lesser extent the articular chondrocytes to modify their surrounding matrix. The cells in the nasal cartilage appear less susceptible. Interestingly, the pattern of matrix constituents are more similar between epiphyseal and articular cartilage than between these and nasal cartilage (Paulsson and Heinegård, 1982). It is possible that the chondrocytes have a different phenotype showing different sensitivity to the factors involved. Whether or not the proteases implied are made by the chondrocytes or provided from external sources is not known. Since the central epiphyseal cartilage is the prime target for the increased catabolism rather than the surrounding articular cartilage it appears, however, that the source is not external, but rather from within the cartilage. Possible the delayed elimination of proteoglycans from the articular cartilage, may result from a diffusion of proteases from the epiphyseal cartilage.

The drastic changes in proteoglycan turnover at the time of calcification are strong indications that these molecules play important roles in the process.

Variations in the composition of bovine hip articular cartilage with distance from the articular surface (III).

The calcification process in the rat femoral head (i.e. proximal epiphysis) does not extend beyond a certain region in the epiphysis, sparing the articular cartilage uncalcified in the periphery. Today, the factors that make the articular cartilage matrix resistant to calcification throughout life are far from known. One such factor that may be involved are the proteoglycans. The present investigation was therefore initiated to determine whether the articular cartilage proteoglycans vary with distance from the joint surface and become more similar to those of the calcifying cartilage near the osteochondral region, where the calcification process has stopped. To obtain sufficient quantity of articular cartilage and to avoid the problem of interindividual variation, bovine femoral head cartilage was chosen for the study. A cartilage-bone cylinder was prepared from the femoral head of a bull. Three main regions were morphologically identifiable in the articular cartilage. One region close to the joint cavity i.e.

the superficial layer, contained flattened fibroblastic like cells, positioned in a fibrillar structure showing relatively little metachromasia when stained with toluidine blue. The deepest region, a narrow region at the osteochondral border, contained spheriodal chondrocytes surrounded by a calcified cartilaginous matrix. The middle region showed the typical appearance of a cartilage matrix with few chondrocytes scattered in an extracellular matrix showing few structures and prominent metachromasia. The articular cartilage was freeze-sectioned parallel to the joint surface and several sections were taken down to the osteochondral junction and pooled into groups representing the morphologically different regions of the articular cartilage. Proteoglycans were extracted and characterized using conventional techniques. The middle region, accounting for 70-80% of the cartilage thickness expectedly had a higher content of proteoglycans than the other two regions. It has also been shown by others (Maroudas et al., 1969; Lempberg et al., 1972; Maroudas, 1979) that the content of proteoglycans (quantitated as glycosaminoglycans) was highest in the middle region of articular cartilage. About 90% of the proteoglycans in that region had the ability to form aggregates with hyaluronic acid. Furthermore, the proteoglycans had a higher content of protein and a lower content of chondroitin sulphate compared with those in the deep region. The deepest region representing some 10 % of the total had a relatively lower content of chondroitin sulphate rich proteoglycans. Furthermore, a lower proportion of these proteoglycans could form aggregates with hyaluronic acid compared with those in the two other regions. The superficial region, accounting for about 10-15% of the total articular cartilage, contained quite different small proteoglycans which all appeared to be able to form aggregates. The protein content of these small proteoglycans was higher than for proteoglycans from the other regions of the cartilage. The average size of the glycosaminoglycan chains was identical in the proteoglycans from the three regions. In a recent study (Bayliss et al., 1983) obtained similar results with human articular cartilage obtained both at autopsy and surgery. They showed that the proteoglycan aggregatability was quite high (about 70% of total) in all layers, with the exception of those from the osteochondral border, where it was lower. These authors, however, were unable to identify a population of small proteoglycans in the uppermost layers (paper III). It is possible that species or age differences may explain the discrepancy. The articular cartilage from the bovine source (paper III) was from young animals, while the human cartilage was from old individuals.

In conclusion, the calcification process was confined to a region representing about 10-15% of articular cartilage thickness. The proteoglycans from this region differed from those in the uncalcified parts of the cartilage. The calcified region showed a decreased content of proteoglycans with an impaired ability to form aggregates. It appears then, that these differences are very similar in nature to those observed in paper I and paper II and observed by others (Lohmander and Hjerpe, 1975; Vittur et al., 1977; Pita et al., 1979). To obtain further information on the nature of the factors preventing calcification of the middle and surface layers of the articular cartilage, it is probably necessary to expand studies to involve other matrix constituents, like the proteins.

Extraction and purification of proteoglycans from mature bovine bone (IV)

To improve our understanding about molecular transitions during the osteogenesis, the other main event in endochondral ossification, it is important to understand the nature of the processes involved. One important step is to learn about the structure of the macromolecules in bone and how these differ from those in cartilage. Such information would provide important background as to which molecules are eliminated and what other types of molecules are synthesized. As discussed above proteoglycans are important constituents of the extracellular matrix and those from cartilage are fairly well characterized. Therefore, for comparison those from bone were extracted, purified and characterized. Particular attention was paid to the isolation of only those proteoglycans found in the mineral matrix of the bone, thereby avoiding those present in blood vessels and other tissues present. For this purpose proteoglycans not bound to mineral were first extracted from powderized bone with 4M guanidine hydrochloride (GuHCl-extract). The efficiency of the extraction was monitored by using proteoglycans from blood vessel wall as a marker. After about 6 hrs, the extraction efficiency of these proteoglycans had reached a maximum value. The residue was subsequently demineralized and extracted with 4M guanidine hydrochloride containing EDTA (GuHCl-EDTA-extract). The initial GuHCl-extract accounted for about 8 per cent, and the second GuHCl-EDTA-extract for about 60 per cent of the total proteoglycans in the bone. Those in the second extract did not react in the immunoassay used to quantitate vessel wall proteoglycans and their glucosaminoglycan to galactosaminoglycan ratio was lower indicating that they differ from those proteoglycans re-

covered in the first extract. Only those proteoglycans extracted from the mineral phase were purified further. Interestingly, although extractions were done with extreme precautions to prevent proteolysis, the GuHCl-EDTA extract contained both proteoglycans and glycosaminoglycan peptide chains, as shown by agarose-polyacrylamide gel electrophoresis. It is likely, however, that this results from *in vivo* processing. The bone proteoglycans were purified by CsCl density gradient centrifugation in 4 M GuHCl and by Sepharose CL4B chromatography. The glycosaminoglycan peptides, having a higher buoyant density, could then be removed in a second CsCl density gradient centrifugation. The bone proteoglycans were further purified to homogeneity by DE52 anion exchange chromatography to remove small amounts of sialoproteins and subsequently by hydroxyapatite chromatography. The last step gave three populations of bone proteoglycans having different elution positions and different carbohydrate to protein contents. Furthermore, their amino acid composition differ and interestingly the size of the chondroitin sulphate side chains differ, most notably also from that of the glycosaminoglycan peptides present in the original extract. It appears, therefore that those chains originate from a different proteoglycan than that typical for bone. The predominating proteoglycan representing about 70 % had the ability to selfassociate in buffers not containing 4M guanidine hydrochloride or 7M urea. The aggregation was abolished when the proteoglycan core protein was destroyed by alkaline treatment indicating that the aggregation was mediated mainly by the proteoglycan core protein alone or in combination with the glycosaminoglycan chains of another molecule.

The molecular characteristics observed for the bone proteoglycan in the present study corroborate and extend previous data showing that the major bone proteoglycan is small and protein rich (Engfeldt and Hjerpe, 1976; Reddi et al., 1978; Fischer et al., 1983a) and has large glycosaminoglycan side chains (Reddi et al., 1978; Fischer et al., 1983a). Only Fischer et al. (1983a), however, used a technique similar to the one used in the present study, which allows the isolation of only those proteoglycans bound in the bone mineral matrix. Their data, obtained with a preparation from subperiosteal bone of young calves, are very similar to those in paper III and IV, i.e. the amino acid composition is similar, the proteoglycans chromatograph retarded even on 4 % agarose gels and they contain a core protein of similar apparent molecular weight. There are, however, some compositional differences perhaps due to the different age of the animals. The major proteoglycan described in paper III and IV has almost twice the

protein content and only about one third of the sialic acid content of that of the preparation characterized by Fischer et al. (1983a). It is possible, however, that this discrepancy depends on the presence of some glycosaminoglycan peptides in the preparation characterized by these authors. The techniques they use do not separate proteoglycans from glycosaminoglycan peptides, if present.

Characterization of proteoglycans from the calcified matrix of bovine bone (V)

The main proteoglycan isolated from the mineral matrix of bone was characterized further. Its apparent molecular weight was found to be only 74,600 dalton determined by sedimentation-equilibrium centrifugation in 4M GuHCl and its sedimentation coefficient ($S_{20,w}^0$) was 3.04. This proteoglycan then, has molecular dimensions similar to those of the small proteoglycans in cartilage and several other tissues, but considerably smaller than aggregating cartilage proteoglycans. Furthermore, also the apparent molecular weight of the core protein after chondroitinase digestion to depolymerize the glycosaminoglycan side chains was 45,000 dalton, determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This is typical for the small proteoglycans as are the high contents of aspartic acid/asparagine, glutamic acid/glutamine and leucine (Axelsson and Heinegård, 1980; Cöster and Fransson, 1981; Heinegård et al., 1981a; Fischer et al., 1983a). A more true molecular weight of the protein core, however, is about 30,000 dalton, as estimated from the protein content and the molecular weight of the proteoglycan. It should be remembered that the core protein prepared by chondroitinase digestion still contains a number of oligosaccharides, possibly explaining its anomalously high molecular weight calculated from SDS-PAGE. The molecular weight of the glycosaminoglycan side chains, determined by Superose 6B chromatography after the proteoglycan core protein was destroyed by alkaline-borohydride treatment was 18,579 and 33,677 dalton for Mn and Mw, respectively. Consequently also these proteoglycans contain the large, very polydisperse glycosaminoglycan side chains found in many other small proteoglycans (Heinegård and Paulsson, 1984). These chains were chondroitin sulphate, since they were completely depolymerized by digestion with chondroitinase ACII. Additional side chain substituents were several types of oligosaccharides, both large, probably linked via N-glycosidic linkage to asparagine, and small, linked via a O-glycosidic linkage to serine or threonine.

Taken together the data indicate that the major bone proteoglycan is a protein rich molecule containing a core protein having one or two chondroitin sulphate chains substituents plus several oligosaccharides. This representation of the molecule is similar to that of the cartilage, small proteoglycan (Heinegård et al., 1981a). Further information on the structural similarity was obtained by determination of immunological crossreactivity by using enzyme linked immunosorbent assay of the bone as well as of the cartilage, small proteoglycans. In spite of the similar composition of the two molecules, the immunoassay showed only partial crossreactivity, indicating that the molecules contained some regions of similar structure, while other regions were dissimilar. Further studies have indicated that the bone proteoglycan is more similar to small proteoglycans of fibrous tissues like sclera, tendon and cornea (unpublished), although the side chains of these proteoglycans are dermatan sulphate.

The antibodies against the bone proteoglycan was also used for immunolocalization. Most of the immunoreactivity for bone proteoglycans (i.e. immunofluorescence) was observed in the region of active osteogenesis (primary spongiosa). No immunofluorescence was detected among the hypertrophied chondrocytes or in the region of provisional calcification.

Zonal rate centrifugation of proteoglycans in sucrose gradients (V)

Since the proteoglycan aggregates are among the largest molecules in the body, methods available for fractionating them according to size are few and often rely on chromatographic procedures, where losses may occur due to nonspecific adsorption to the matrix used. The present investigation was initiated to develop suitable conditions for separation of proteoglycan aggregates, proteoglycan monomers and fragments thereof by zonal rate centrifugation in preformed sucrose gradients thereby creating a separation in free solution with few effects of a support matrix. Such gradients have been successfully used by others (Kato et al. 1978; Vasan and Lash, 1979) to fractionate isotope-labelled proteoglycan, but no attempts have been made to optimize conditions for different types of separations or to allow demonstration of sedimentation properties of non-labelled components. The use of sucrose has many advantages, like not being expensive and potentials for use in mixtures with different types of buffers and pH. It has a low absorbance even at low wavelengths like 206 nm where groups like the N-acetyl of the glycosaminoglycans absorb UV-light. The absorbance at 206 nm was therefore used for very sensitive monitoring of pro-

teoglycans or fragments thereof and the UV-profiles were found to be almost identical to those for the distribution of proteoglycan protein and hexosamine. The sedimentation rate of the proteoglycan monomers was studied as a function of sucrose concentration (10-30 % and 10-50 %), the amount of the sample centrifuged and the ionic environment in the gradient. As expected the sedimentation rate in the 10-30% gradient was higher than in the 10-50% gradient. Furthermore, the sedimentation in the 10-30% gradients was isokinetic i.e. the molecules sedimented with a constant velocity over the tube length. The 10-30% gradient was found suitable for separation of proteoglycan monomers or fragments thereof, while the 10-50% gradient was best suited for separation of the more polydisperse proteoglycan aggregates, where the high concentration of sucrose in the bottom of the centrifuge tube slowed the sedimentation of the largest aggregates. By changing the ionic environment in the gradient it was possible to manipulate the sedimentation rate of the proteoglycans in a different manner compared with the proteins. For example, the sedimentation rate of proteoglycan monomers in the presence of 0.5 M cesium chloride was almost twice that observed in the presence of 0.5 M sodium chloride. Furthermore, the sedimentation of these molecules in the presence of 5 mM phosphate was extremely slow because of the extended structure of the proteoglycans. The technique was also suitable for rapid separations of proteoglycan aggregates from monomers. Indeed, at the same time the size distribution of the aggregates was obtained. For example, the aggregates from the nasal cartilage sedimented with a higher rate than those from the tracheal and articular cartilages, while the monomers from these tissues showed similar sedimentation rates. These results are consistent with those obtained by Hascall and Heinegård (1974b), who observed that proteoglycan aggregates from nasal cartilage were larger than those from tracheal cartilage, i.e. 93 and 43, respectively. The presently described procedure for fractionation of proteoglycans has certain advantages compared with zonal rate centrifugation in CS_2SO_4 gradients described by Kimata et al. (1982). Thus, the conditions of the gradient, i.e. types and concentrations of counterions can be changed over a wide range in the sucrose.

CONCLUDING REMARKS

One of the two major events in endochondral ossification is the calcification process, and the other is the osteogenesis, which is initiated only at the surface of calcified cartilage remnants (for ref. see Ham and Cormack, 1979). The calcification is preceded by a hypertrophy of the chondrocytes and subsequent removal of the cartilage proteoglycan aggregates (Pिता et al. 1979, paper II). In the growth and development of the rat femoral head, the hypertrophy seems to be a specific feature of the epiphyseal chondrocytes (Koshino, 1975). Although the articular cartilage is in a close contact with the epiphyseal cartilage, its chondrocytes are virtually unaffected. One may speculate on what are the factors, local or/and systemic, that control this event. It is possible that the hypertrophy is initiated when the chondrocytes in the middle of the femoral head have reached a critical cell density. Alternatively, the epiphyseal chondrocytes might already be programmed to start to hypertrophy at a certain age of the animal or hypertrophy could be induced by locally or systemically produced factors of unknown nature. It is thought, that even if the chondrocytes of both articular and epiphyseal cartilages have the same precursor cell, i.e. the mesenchymal cell, they early develop characteristics typical for epiphyseal and articular cartilages, respectively. McKibbin and Holdsworth (1967) in an elegant experiment, prepared a cartilaginous cylinder from a growing femoral head, turned it 180 degrees and subsequently put it back in the femoral head with the epiphyseal portion now near the surface. They observed that during subsequent growth and development of the femoral head the specimen corresponding to the presumptive, original articular cartilage did not calcify, even though the surrounding epiphyseal cartilage did. Thus, the differentiation into chondrocytes programmed to form epiphyseal and articular cartilage, respectively, appears to occur very early in the skeletogenesis. It is still possible that the epiphyseal chondrocytes produce factors, which at a certain stage can initiate the hypertrophy of these cells, while the chondrocytes in the articular cartilage do not respond to such factors.

Concomitantly with hypertrophy of the epiphyseal chondrocytes and subsequent calcification, proteoglycans were eliminated from the epiphyseal cartilage at an increased rate (paper II). Furthermore, some 3 to 4 days later, similarly the proteoglycans in the articular cartilage showed an increased elimination rate, while the non-related nasal cartilage showed no such changes. The increased elimination rate in only the epiphyseal and

the articular cartilages with the time lag of about 3-4 days, indicates that some factor produced in the epiphyseal cartilage may diffuse into the articular cartilage and also there initiate proteoglycan degradation. Proteoglycan aggregates are considered to be potent inhibitors of calcification (for ref. see Buckwalter, 1983). It is consistent then, that to promote calcification, these aggregates are removed as described in paper II. The exact nature of the process is unknown, but it is likely that proteases are involved. One should remember that the effects of the increased catabolism are reinforced by the contemporary slower biosynthesis rate. Thus, the factors involved have more than one effect on the chondrocytes. What is the nature of the factor or factors? Interestingly, a comparative study of the protein patterns in the epiphyseal and articular cartilages of the rat femoral head, revealed that the ongoing calcification was accompanied with the appearance in the epiphyseal cartilage of an unidentified protein of low molecular weight (Franzén and Heinegård, to be published). It is possible that this protein represents one factor involved. To obtain information on the other major process, i.e. osteogenesis, the proteoglycans present in the calcified bone matrix were isolated and characterized (paper IV, V). The molecules compared with those in calcified cartilage matrix were distinctly different, probably representing altogether different molecular entities. Furthermore, it is well known that while cartilage contains type II collagen, that of bone is type I. Therefore, it appears that the chondrocyte of calcified cartilage is replaced by another cell of mesenchymal origin, having a very different biosynthetic capacity. Again the factors involved are little known, but quite important for better understanding of processes like fracture healing, where the events observed in the femoral head are repeated. Increased knowledge of the molecules specific for bone and involved in bone formation and repair would also improve our understanding and ability to interfere with other medical problems like osteoporosis and joint diseases, in particular rheumatoid arthritis where bone involvement is an early feature.

ACKNOWLEDGEMENTS

This investigation had not been fulfilled without the help of so many people. I want to express my sincere gratitude to some of them:

Dick Heinegård, my chief, advisor and friend, who brought me into the fascinating field of connective tissue research. I have had the privilege so many years of working with him at the research front of skeletal tissue proteoglycans. During these years, his continuous and never failing encouragement and constructive criticism have been invaluable for the performance of present investigation.

Sven Gardell, who a snowy and cold Sunday in January 1971, with great kindness and enthusiasm introduced me as a young student to real biochemical science.

Sigvard Kopp and Maria Nilner for their continuous interest and encouragement.

Lars Hammarström, for his generous support and who introduced me into the field of morphological research.

Staff members at the Faculty of Odontology in Malmö, especially Per-Olof Glantz, Stig Edwardsson and Downen Birkhed for their generous support.

Sara Axelsson, Annika Björne-Persson, Britt Mattsson, Ros-Marie Sandfalk Kristina Roslund and Inger Wännman for skilful technical assistance.

Marianne Larsson and Ruth Lovén who carefully and patiently typed the manuscripts and the thesis.

Birgitta Jönsson for skilfully drawing all the figures and Lars Seiron for carefully preparing the photographic material.

Berne Eriksson, Gunborg Bülow and Tosca Engel for excellent librarial assistance.

Runo Svensson for technical expertise and ingenious constructions.

Brita Lagergren who kindly supplied me with information about available jobs and grants throughout these years.

All the staff and personnel at the Departments of Physiological Chemistry and Stomatognathic Physiology, University of Lund, for their encouragement and friendly support.

Finally, I wish to thank my wife, Monica, who patiently took care of our home and our children Henrik and Malin, while I frequently stayed at the laboratory until the middle of the night. I therefore dedicate this thesis to them.

This investigation was supported by grants from the Swedish Medical Research Council (05668), Folksams Yrkesskadors Stiftelse, Kock's Stiftelser, Österlunds stiftelser, Konung Gustaf V:s 80-års fond and from The Faculties of Medicine and Odontology, University of Lund.

REFERENCES.

- Anderson, H.C. (1980) Path. Ann. 15, 45-75
- Ashton, B.A., Höhling, H.J. and Triffitt, J.T. (1976) Calcif. Tiss. Res. 22, 27-33
- Axelsson, I. and Heinegård, D. (1980) Exp. Eye Res. 31, 57-66
- Bayliss, M.T., Venn, M., Maroudas, A. and Ali, S.Y. (1983) Biochem. J. 209, 387-400
- Björnsson, S. and Heinegård, D. (1981) Biochem. J. 199, 17-29
- Bloom, W. and Fawcett, D.W. A textbook of histology. 10th ed. Philadelphia Saunders, 1975, pp. 262-279
- Blumenthal, N.C., Posner, A.S. and Rosenberg, L.C. (1980) Trans. Orthop. Res. Soc. 5, 10
- Boskey, A. L. (1981) Clin. Orthop. 157, 225-257
- Boström, H. (1952) J. Biol. Chem. 196, 477-81
- Buckwalter, J. A. (1983) Clin. Orthop. 172, 207-232
- Cecil, R. N. A. and Anderson, H. C. (1976) Metab. Bone Dis. Rel. Res. 1, 85-95
- Christner, J., Brown, M., and Dziewiatkowski, D. (1978) Anal. Biochem. 90, 22-32
- Cuervo, L. A., Pita, J.C. and Howell, D. S. (1973) Calcif. Tiss. Res. 13, 1-10
- Cöster, L. and Fransson, L.-Å. (1981) Biochem. J. 193, 143-153
- DeLuca, S., Lohmander, S., Nilsson, B., Hascall, V.C. and Caplan, A.J. (1980) J. Biol. Chem. 255, 6077-6083
- Engfeldt, B. and Hjerpe, A. (1976) Acta Pathol. Microbiol. Scand. 84, 95-106
- Faltz, L. L., Caputo, C. B., Kimura, J. H., Schrode, J. and Hascall, V. C. (1979) J. Biol. Chem. 254, 1381-1387
- Fellini, S.A., Kimura, J.H. and Hascall, V.C. (1981) J. Biol. Chem. 256, 7883-7889
- Fisher, L. W., Termine, J. D., Deijter, S. W., Whitson, S. W., Yanagishita, M., Kimura, J. H., Hascall, V. C., Kleinman, H. K., Hassel, J. R. and Nilsson, B. (1983a) J. Biol. Chem. 258, 6588-6594
- Fisher, L. W., Whitson, S. W., Avioli, L. V. and Termine, J. D. (1983b) J. Biol. Chem. 258, 12723-12727
- Franek, M. D. and Dunstone, J. R. (1967) J. Biol. Chem. 242, 3460-3467
- Franzén, A., Björnsson, S. and Heinegård, D. (1981) Biochem. J. 197, 669-674

- Granda, J. L. and Posner, A. S. (1971) Clin. Orthop. 74, 269-272
- Ham, A. W. and Cormack, D. H. Histology 8th ed., J. B. Lippincott Co, Philadelphia, Toronto, 1979, pp. 377-467
- Hardingham, T. E. (1979) Biochem. J. 177, 237-247
- Hardingham, T. E. and Muir, H. (1972) Biochim. Biophys. Acta 279, 401-405
- Hascall, V. C. and Heinegård, D. (1974a) J. Biol. Chem. 249, 4232-4241
- Hascall, V. C. and Heinegård, D. (1974b) J. Biol. Chem. 249, 4242-4249
- Hascall, V. C. and Riolo, R. L. (1972) J. Biol. Chem. 247, 4529-4538
- Hascall, V. C. and Sajdera, S. W. (1970) J. Biol. Chem. 245, 4920-4930
- Hauschka, P.V., Lian, J.B. and Gallop, P.M. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3925-3929
- Heinegård, D. and Axelsson, I. (1977) J. Biol. Chem. 252, 1971-1979
- Heinegård, D. and Hascall, V. C. (1974a) J. Biol. Chem. 249, 4250-4256
- Heinegård, D. and Hascall, V. C. (1974b) Arch. Biochim. Biophys. 165, 427-441
- Heinegård, D. and Hascall, V.C. (1979a) J. Biol. Chem. 254, 921-926
- Heinegård, D. and Hascall, V. C. (1979b) J. Biol. Chem. 254, 927-934
- Heinegård, D. and Paulsson, M. (1984) In Connective Tissue Biochemistry. Piez, K. and Reddi, H.A.,(eds)., Elsevier Science Publishing, New York, pp. 277-328
- Heinegård, D., Paulsson, M. and Wieslander, J. (1981a) Sem. Arthr. Rheum., suppl. 1, 31-33
- Heinegård, D., Paulsson, M., Inerot, S. and Carlström, C. (1981b) Biochem. J. 197, 355-366
- Herring, G. M. (1968) Biochem. J. 107, 41-49
- Herring, G. M. and Kent, P. W. (1963) Biochem. J. 89, 405-414
- Hirschman, A. and Dziewiatkowski, D. D. (1966) Science, vol. 154, 393-395
- Howell, D.S., Pita, J.C., Marquez, J.F. and Madruga, J.E. (1968) J. Clin. Invest. 47, 1121-1132
- Howell, D.S. and Pita, J.C. (1976) Clin. Orthop. 118, 208-229
- Hult, A. (1958) Acta Orthop. Scand. 28, 1-21
- Kato, Y., Kimata, K, Ito, K., Karasawa, K. and Suzuki, S. (1978) J. Biol. Chem. 253, 2784-2789
- Kempson, G. E., Muir, H., Swanson, S. A. V. and Freeman, M. A. R. (1970) Biochim. Biophys. Acta 215, 70-77
- Kimata, K., Barrach, H.-J., Brown, K. S. and Pennypacker, J. P. (1981) J. Biol. Chem. 256, 6961-6968

- Kimata, K., Kimura, J.H., Thonar, E.J., Rennard, S.J. and Hascall, V.C. (1982) J. Biol. Chem. 257, 3819-3826
- Kimura, J. H., Hardingham, T. E., Hascall, V. C. and Solursh, M. (1979) J. Biol. Chem. 254, 2600-2609
- Koshino, T. (1975) Acta Radiol. Suppl. 344, 33-46
- Koshino, T. and Olsson, S.-E. (1975) Acta Radiol. Suppl. 344, 46-52
- Kuettner, K. E., Eisenstein, R., Soble, L. W. and Arsenis, C. (1971) J. Cell Biol. 49, 450-458
- Kuettner, K., Sorgente, N., Croxen, R., Howell, D.S. and Pita, J.C. (1974) Biochem. Biophys. Acta 372, 335-344
- Lempberg, R.K., Larsson, S.E. and Hjertquist, S.-O. (1972) Calcif. Tiss. Res. 15, 253-267
- Lindenbaum, A. and Kuettner, K. (1967) Calcif. Tiss. Res. 1, 153-165
- Lohmander, S. and Hjerpe, A. (1975) Biochim. Biophys. Acta 404, 93-109
- Lohmander, S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B. Kimura, J. H. and Heinegård, D. (1980) J. Biol. Chem. 255, 6084-6091
- Maroudas, A. (1979) In Adult Articular Cartilage. (Freeman M.A.R. ed.) 2nd ed. Pitman, London, pp. 215-226
- Maroudas, A., Muir, H. and Wingham, J. (1969) Biochem. Biophys. Acta 177, 492-500
- McDevitt, C.A., Billingham, M.E. and Muir, J.H. (1981) Sem. Arthr. Rheum. 11, 17-18
- McKibbin, B. and Holdsworth, F. (1967) J. Bone Joint. Surg. 49B, 351-361
- Miller, E.J. and Martin, G.R. (1968) Clin. Orthop. 59, 195-232
- Miller, E. J. and Matukas, V. J. (1969) Proc. Natl. Acad. Sci. U. S. A. 64, 1264-1268
- Mitchell, N., Shepard, N. and Harrod, J. (1982) J. Bone Joint. Surg. 64 A, 32-38
- Muir, H. (1980) In The joints and synovial fluid, vol. III, Solokoff, L. (ed.), Acad. Press, New York, pp. 27-94
- Nieduszynski, J., Sheehan, J., Phelps, C., Hardingham, T. and Muir, H. (1980) Biochem. J. 185, 107-117
- Nilsson, B., DeLuca, S., Lohmander, S. and Hascall, V. C. (1982) J. Biol. Chem. 257, 10920-10927
- Oegema, T.R., Bradford, D.S. and Cooper, K.M. (1979) J. Biol. Chem. 254, 10579-10581
- Ogden, J. A. (1979) In The scientific basis of orthopaedics. Albright, J.A. and Brand, R.A. (eds.) Appelton-Century-Crofts, New York, pp. 41-103

- Orkin, R., Pratt, R. and Martin, G.M. (1976) Dev. Biol. 50, 82-94
- Paulsson, M. and Heinegård, D. (1979) Biochem. J. 183, 539-545
- Paulsson, M. and Heinegård, D. (1982) Biochem. J. 207, 207-213
- Paulsson, M. and Heinegård, D. (1984) Coll. Rel. Res. 4, 219-229
- Perkins, S.J., Miller, A., Hardingham, T.E. and Muir, H. (1981) J. Mol. Biol. 150, 69-95
- Pita, J. C., Muller, F. J. and Howell, D. S. (1975) In Dynamics of connective tissue macromolecules. Burleigh, P.M.C. and Poole, A.R. (eds.) New York, Elsevier, pp. 247-258
- Pita, J. C., Muller, F. J., Morales, S. M. and Alarcon, E. J. (1979) J. Biol. Chem. 254, 10313-10320
- Poole, A.R., Pidoux, J. and Rosenberg, L. (1982) J. Cell Biol. 92, 249-260
- Price, P. A., Otsuka, A. S., Posner, J. W., Kristaponis, J. and Raman, N. (1976) Proc. Natl. Acad. Sci. U. S. A., 73, 1447-1451
- Reddi, A. H. (1981) Coll. Rel. Res. 1, 227-237
- Reddi, A. H., Hascall, V. C. and Hascall, G. K. (1978) J. Biol. Chem. 253, 2429-2436
- Reinholt, F. P. (1983) The Normal and Rachitic Epiphyseal Growth Plate. Doctorial dissertation, Karolinska Institutet, Stockholm
- Rodén, L. (1980) In The Biochemistry of Glycoproteins and Proteoglycans, Lennarz, W.J. (ed.) Plenum Press, New York, pp. 267-371
- Sajdera, S. W. and Hascall, V. C. (1969) J. Biol. Chem. 244, 77-87
- Sapolsky, A., Malemud, C., Norby, D., Moskowitz, W., Matura, K. and Howell, D.S. (1981) Biochim. Biophys. Acta 658, 138-147
- Shuttleworth, A. and Veis, A. (1972) Biochim. Biophys. Acta 257, 414-420
- Slavkin, H. (1979) Developmental craniofacial biology. Lee and Febiger, Philadelphia, pp. 177-234
- Spector, A. R. and Glimcher, M. J. (1972) Biochim. Biophys. Acta 263, 593-603
- Tang, L., Pal, S., Choi, H., Poole, A., Haberman, E. and Rosenberg, L. (1981) Trans. Orthop. Res. Soc. 6, 107
- Tebor, G.B., Ehrlich, M.G., Armstrong, A. and Mankin, H.J. (1982) Trans. Orthop. Res. Soc. 7, 54
- Tengblad, A. (1981) Biochem. J. 199, 297-305
- Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. L. and Martin, G. R. (1981) Cell. 26, 99-105
- Thyberg, J. (1974) J. Ultrastruct. Res. 46, 206-218

- Uldbjerg, N., Malmström, A., Ekman, G., Sheehan, J., Ulmsten, U. and Wingerup, L. (1983) Biochem. J. 209, 497-503
- Vasan, N.S. and Lash, J.W. (1979) J. Embryol. Exp. Morph. 49, 47-59
- Vittur, F., Zanetti, M., Stagni, N. and deBernard, B. (1977) Bull. Mol. Med. 2, 189-198
- Wasteson, Å. (1971) J. Chromatogr. 59, 87-97
- Weatherell, J. A. and Weidman, S. M. (1963) Biochem. J. 89, 265-267
- Weiss, R. E. and Reddi, A. H. (1981) In The chemistry and biology of mineralized connective tissues. Veiss, A. (ed.) Elsevier, North Holland, pp. 607-612

Proteoglycans and Calcification of Cartilage in the Femoral Head Epiphysis of the Immature Rat*

BY AHNDERS FRANZÉN, D.D.S.†, DICK HEINEGÅRD, M.D.†, LUND, SVEN REILAND, V.M.D.‡, AND STEN-ERIK OLSSON, M.D., V.M.D.‡, STOCKHOLM, SWEDEN

From the Department of Physiological Chemistry, University of Lund, Lund, and the Laboratory for Comparative Orthopaedics, National Veterinary Institute, Stockholm

ABSTRACT: The femoral heads of young rats have been used to monitor changes in proteoglycan structure during growth and endochondral ossification. Proteoglycans were extracted in good yield. The tissue content of proteoglycans increased until the time of calcification and then decreased. In contrast, the collagen content increased over the period studied. On Day 20, just preceding the onset of calcification, the proteoglycans had a lower glycosaminoglycan content, were somewhat smaller, and contained a larger proportion of molecules that were not capable of interacting with hyaluronic acid. On Day 25, during ongoing calcification, the proportion of proteoglycans that were not capable of interacting with hyaluronic acid was low, while it again was high on Day 40, just preceding ossification. The relative glycosaminoglycan content of the proteoglycans was somewhat lower on Day 20 and Day 40.

The results indicate that both at the time of calcification and at the time of ossification the proteoglycan structure changes, perhaps indicating a functional role for the proteoglycans in the calcification process.

CLINICAL RELEVANCE: Several crucial biological processes occur in the growth plate during endochondral ossification. The mechanisms involved in these processes in the normal growth plate must be understood before derangements in these processes which occur in growth disorders such as chondrodysplasia can be elucidated. This study examined the role of proteoglycans during normal endochondral ossification.

Most studies of the molecular changes during endochondral ossification have been hampered by the difficulty of isolating tissue specimens representing different stages of the process.

In humans and the domesticated animals, the different stages of endochondral ossification — cell death, matrix calcification, vascular invasion, and bone formation — are temporally close in sequence or overlap. This is

particularly true for the growth plate, in which calcification of the matrix seems to occur just before vascular invasion. Different stages of the process also occur in close proximity spatially in small specimens. These factors make it extremely difficult to study the biochemical events involved in the process of cartilage matrix calcification.

Many investigators therefore have turned to *in vitro* studies or to biological model systems. Proteoglycans, which have a very large number of negatively charged groups, may be involved in the calcification process. A number of studies have therefore been directed to the role of proteoglycans. Pita et al. showed that the amount of proteoglycan aggregates was small in aspirated interstitial fluid from the calcification front of the growth plate of immature rats. Howell et al. and Blumenthal et al. showed that proteoglycan aggregates *in vitro* can inhibit precipitation and growth of amorphous calcium phosphate crystals from saturated solutions. Reddi and Huggins have contributed much information about regulating factors and what events take place using subcutaneous implantation of demineralized bone powder in rats. With time a cartilage nodule is formed, which is calcified and is transformed into bone. Proteoglycans from the different stages of the process have been isolated and characterized²¹, but no significant changes were observed in the proteoglycan structure during calcification of the cartilage matrix.

The aim of the present study was to investigate the alterations in the proteoglycan structure before and during calcification of cartilage using a normal physiological system. The femoral head (proximal epiphysis) of the immature rat was chosen. Koshino¹⁶ has shown that there is a considerable lag period between calcification and ossification in this structure. Calcification begins in the female rat at the age of twenty-one days, with a delay of one day in the male¹⁷. Koshino noted that invasion of vessels and ossification does not begin until about three weeks after the onset of calcification; that is, shortly after Day 40.

Materials and Methods

Six mature female rats and two male rats of the Wistar strain were obtained from Møllegaards Avelslab. (Copenhagen, Denmark) and were bred. Trypsin (type XI, DPCC treated, catalog number TO134) and papain (type III, 2X crystallized, catalog number P3125) were purchased from Sigma Chemical (U.S.A.). Sepharose CL2B and CL6B, and Sephacryl S-200, were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Aristar hydrochloric acid was obtained from BDH (Poole, England). All other chemicals used were of analytical grade. High-molecular-weight rooster-comb hyaluronic acid was a gift from Pharmacia AB (Uppsala, Sweden).

* Supported by grants from the Swedish Medical Research Council (13P-5739 and 13X-05668), Alfred Österlunds Stiftelse, Kocks Stiftelse, Konung Gustaf V:s 80-års Fond, and the Medical and Odontological Faculties, University of Lund.

† Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden.

‡ Laboratory for Comparative Orthopaedics, National Veterinary Institute, Roslagsvägen 101, S-104 05 Stockholm, Sweden.

Tissue Preparation

Rats at the ages of ten, fifteen, twenty, twenty-five, and forty days and of both sexes were obtained from twelve litters. The number of animals in each age group was sixteen, fifteen, nineteen, thirteen, and sixteen, respectively. The animals were anesthetized with ether and were killed by cervical dislocation. The femora were dissected free from soft tissue. The left proximal femoral epiphysis was separated from the metaphysis, washed in 0.15-molar sodium chloride and 0.005-molar phosphate buffer, pH 7.4, and placed under a dissecting microscope. Remnants of the metaphysis were removed from the epiphyseal cartilage, which was immediately frozen in liquid nitrogen and stored at -60 degrees Celsius until it was examined. In some cases the wet weight of the femoral head was determined immediately after dissection. Animals for studies of one or two age groups were commonly taken from each litter.

Histological Investigation

The right proximal femoral epiphysis from the seventy-nine rats of different age groups was separated from the rest of the femur. The epiphysis was cut longitudinally into two equal parts and was fixed in 4 per cent buffered neutral formaldehyde, to which was added 0.5 per cent cetylpyridinium chloride⁸. It was then embedded in paraffin without previous decalcification and serial histological sections, six micrometers thick, were prepared. The sections were stained with either hematoxylin and eosin or von Kossa's stain.

Proteoglycan and Collagen Content

The collagen and proteoglycan content of the femoral heads was determined using other animals than those used for proteoglycan characterization. Rats from one litter were killed at the indicated ages. The right and left femoral heads were removed and were cleaned of surrounding tissues. The wet weights of the four femoral heads from two animals were measured. The samples were then digested for seventy-two hours at 65 degrees Celsius with papain (twenty microliters of a suspension containing twenty-five milligrams of protein per milliliter) in one milliliter of fifty-millimolar sodium phosphate, pH 6.5, containing five-millimolar disodium EDTA and five-millimolar cysteine hydrochloride¹. The digests were freeze-dried and then redissolved in identical volumes of water. Weighed aliquots were studied by Ectecol-cellulose ion-exchange chromatography². Glycopeptides were eluted from the column with water and 0.02-molar hydrochloric acid. The pure glycosaminoglycans were eluted with two-molar hydrochloric acid. The acid was immediately removed using a Rotamix evaporator. The fractions containing the glycosaminoglycans were hydrolyzed in four-molar hydrochloric acid (Aristar) at 100 degrees Celsius for ten hours in tubes sealed under argon. Hexosamines were separated and quantitated using an automatic amino-acid analyzer.

Other weighed aliquots from the digests were hydrolyzed in six-molar hydrochloric acid for twenty-four hours at 100 degrees Celsius and were used for quantitation of hydroxyproline²².

Isolation of the Proteoglycan (A1 Fraction)

Frozen samples from the left femoral head of all the animals in one litter were pooled and pulverized in liquid nitrogen as described by Inerot et al.¹⁵. The pulverized cartilage samples (about fifty milligrams of wet weight) were extracted with continuous stirring for twenty-four hours at 4 degrees Celsius using three milliliters of four-molar guanidinium chloride in 0.05-molar sodium acetate, pH 5.8, containing the protease inhibitors 0.005-molar benzamide, 0.1-molar 6-amino hexanoic acid, and 0.01-molar EDTA as described by Oegema et al. The extracts and residues were separated by centrifugation in a Sorvall RC2-B centrifuge at 19,000 revolutions per minute and 4 degrees Celsius for twenty minutes using the SE-12 rotor.

The pellets were washed twice with 0.5 milliliter of extraction medium and were centrifuged as just described. The supernatants from the three centrifugation steps were pooled; the pools are henceforth referred to as the cartilage extracts. Weighed aliquots from each extract and the corresponding residue were stored at -20 degrees Celsius and were later used for the determination of extraction yields. The remaining extract was dialyzed against ten volumes of 0.05-molar sodium acetate, pH 5.8, containing the protease inhibitors, and the density was adjusted to 1.64 grams per milliliter by the addition of solid cesium chloride. The extracts were then centrifuged for sixty hours at 12 degrees Celsius and 35,000 revolutions per minute using an MSE ten by ten-millimeter titanium angle rotor and a seven-milliliter tube. After centrifugation, the cartilage tubes were frozen in a vertical position by immersing them in liquid nitrogen. The bottom two-fifths (A1 fraction with a density of 1.71 grams per milliliter) was separated from the rest of the tube with a saw. The densities in the extracts and in the fractions were determined by pycnometry using a 300-microliter constriction pipette. The fractions were dialyzed against fifty volumes of 0.5-molar sodium acetate, pH 7.0, and then extensively against water, and were lyophilized.

Column Chromatography

Analytical Sepharose CL2B and CL6B and Sephacryl S-200 columns (0.3 by 140 centimeters) were eluted with 0.5-molar sodium acetate, pH 7.0, at 4 degrees Celsius. The eluent was pumped with an infusion pump at a constant flow of about 900 microliters per hour and fractions of 300 microliters were collected with a time-controlled fraction collector. The uronic-acid content in each fraction was analyzed using an automated version of the carbazole method⁹.

Chemical Methods

Extraction yields were determined as follows. Residues and aliquots of the corresponding extracts were extensively dialyzed against water and were lyophilized. The samples were transferred to 1.5-milliliter Eppendorf centrifuge tubes (Eppendorf Gerätebau, Hamburg, West Germany) and suspended in 500 microliters of fifty-millimolar sodium phosphate, pH 6.5, containing five-millimolar sodium EDTA and five-millimolar cysteine hydrochloride. Papain (twenty microliters of a suspension containing twenty-five milligrams of protein per milliliter) was added and the samples were digested at 65 degrees Celsius for seventy-two hours¹. The glycosaminoglycan fractions were isolated from the digest as already described. The hexosamine contents of these glycosaminoglycan fractions as well as of the A1 fractions were determined after hydrolysis, as described. The extraction yields were calculated from the amounts of hexosamines found in the glycosaminoglycan fractions isolated from the residues and the extracts.

The amino acid composition and protein contents of the A1 fractions were determined with an automatic amino-acid analyzer after hydrolysis of samples in six-molar hydrochloric acid (Aristar) at 110 degrees Celsius for twenty-four hours in tubes sealed under argon. Hydroxyproline contents were determined using the procedure of Stegemann and Stadler after hydrolysis of samples for twenty-four hours at 100 degrees Celsius in six-molar hydrochloric acid.

Gel Chromatography

In all of the chromatographic experiments to be discussed, 150 micrograms of the various A1 fractions were used.

The proportions of aggregates and monomers were determined by Sepharose CL2B gel chromatography. Prior to chromatography, samples of the A1 fractions were incubated for twelve hours at 4 degrees Celsius with added rooster-comb hyaluronic acid (0.01 weight per proteoglycan dry weight) to ensure binding of all proteoglycan monomers capable of interaction with hyaluronic acid.

Aggregates chromatographed in the void volume, while the monomers not bound to hyaluronate eluted in the included volume^{8,11}. Samples of the various A1 fractions were reduced and alkylated as described previously⁹ to prevent the proteoglycan monomers from forming aggregates with hyaluronic acid.

The size distribution of the proteoglycan monomers was then determined by gel chromatography on a Sepharose CL2B column. In order to monitor the distribution of chondroitin sulphate chains on the core protein, samples of the A1 fractions were digested with trypsin¹⁹. Samples of the A1 fractions were dissolved in 0.1-molar sodium phosphate, pH 7.5, and digested with 0.05 milligram of enzyme per milligram of proteoglycan at 37 degrees Celsius for five hours. The digests were fractionated by Sepharose CL6B gel column chromatography to discern the size of the clusters of chondroitin sulphate chains¹⁰. The sizes of the individual chondroitin sulphate chains in the proteoglycans were determined by chromatography of papain digests on Sephacryl S-200 gel columns. Samples of the A1 fractions were digested for three hours at 65 degrees Celsius with two microliters of papain¹ in fifty-millimolar sodium phosphate, pH 6.5, containing five-millimolar sodium EDTA and five-millimolar cysteine hydrochloride.

Results

Morphological Investigation

Koshino, and Koshino and Olsson, histologically characterized the development of the femoral heads of growing rats. In the present work, only the histological appearance of femoral heads from rats of the age groups used for chemical studies is shown. The following ages were selected: young animals at a stage prior to matrix changes at Day 10 (Figs. 1-A and 1-B); animals at a stage just prior to calcification^{16,17}, when the chondrocytes in the central region of the femoral head were hypertrophic, at Day 20 (Figs. 1-C and 1-D); animals at a stage when calcification had started centrally in the femoral head at Day 25 (Figs. 1-E and 1-F); and animals at a stage when the whole epiphyseal cartilage of the femoral head had calcified and all cells were hypertrophic, at Day 40, just prior to ossification^{16,17} (Figs. 1-G and 1-H). At this time only the articular cartilage was not changed. The changes observed were consistent with those reported previously^{16,17}.

In the present investigation, the composition of the whole femoral head was studied, regardless of the fact that it contains both articular and epiphyseal cartilage. Several reports have shown that the proteoglycan structure of

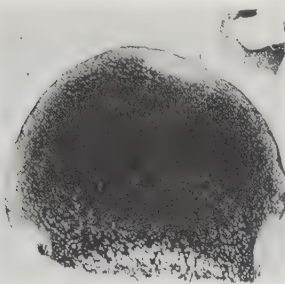


FIG. 1-A

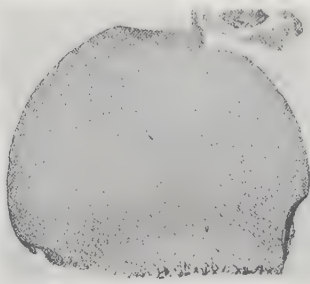


FIG. 1-B

Figs. 1-A through 1-H: Vertical six-micrometer sections taken from femoral heads of female rats of various ages.

Figs. 1-A and 1-B: Ten days old.

Fig. 1-A: The section shows a great number of chondrocytes surrounded by an abundant extracellular matrix (hematoxylin and eosin, $\times 20$).

Fig. 1-B: No inorganic material was observed in the extracellular matrix (von Kossa, $\times 20$).

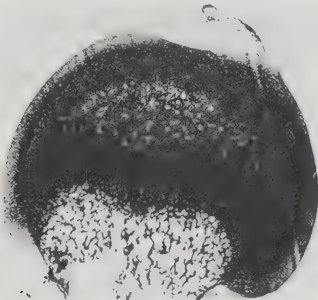


FIG. 1-C

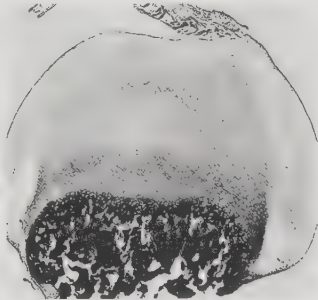


FIG. 1-D

Figs. 1-C and 1-D: Twenty days old.

Fig. 1-C: The chondrocytes in the central portion of the epiphyseal cartilage have begun to hypertrophy (hematoxylin and eosin, $\times 20$).

Fig. 1-D: The calcification process has not yet started (von Kossa, $\times 20$).

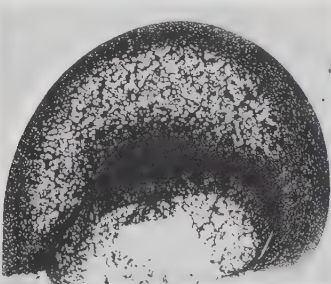


FIG. 1-E

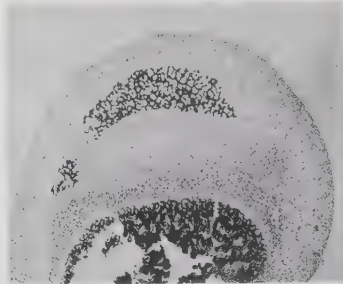


FIG. 1-F

Figs. 1-E and 1-F: Twenty-five days old.

Fig. 1-E: The region in the epiphyseal cartilage with hypertrophic chondrocytes has increased. Two different types of cartilage could be demonstrated in the femoral head: articular and epiphyseal (hematoxylin and eosin, $\times 20$).

Fig. 1-F: Inorganic material has been deposited in the central portion of the epiphyseal cartilage (von Kossa, $\times 20$).

hyaline cartilage changes relatively little with age^{3,14,15}, and the changes observed were of a different character than those that we noted. It is therefore likely that any changes observed in the whole femoral head result from processes in the epiphysis rather than in the articular cartilage.

Proteoglycan and Collagen Contents

As an indicator of the growth of the rats, the wet

weights of their femoral heads increased in an almost linear fashion between the ages of ten and forty days (Fig. 2). The content of collagen (hydroxyproline) in each femoral head also increased with age, almost parallel to the increased weight (Figs. 2 and 3). Therefore, the collagen content can be taken as an indicator of growth. The proteoglycan content in each femoral head, measured as glycosaminoglycan-hexosamine, also increased in an almost linear fashion between the ages of ten and twenty-

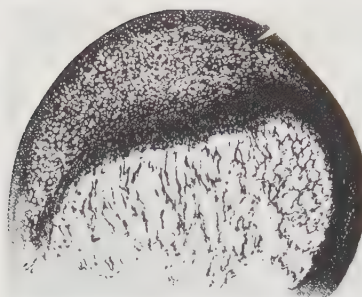


FIG. 1-G

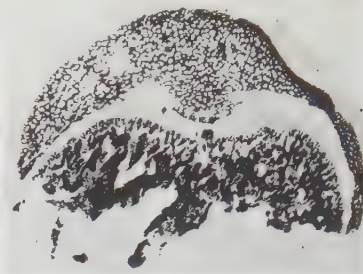


FIG. 1-H

Figs. 1-G and 1-H: Forty days old.

Fig. 1-G: The whole epiphyseal cartilage in the femoral head is occupied by hypertrophic chondrocytes (hematoxylin and eosin, $\times 20$).

Fig. 1-H: The whole femoral head is calcified (von Kossa, $\times 20$).

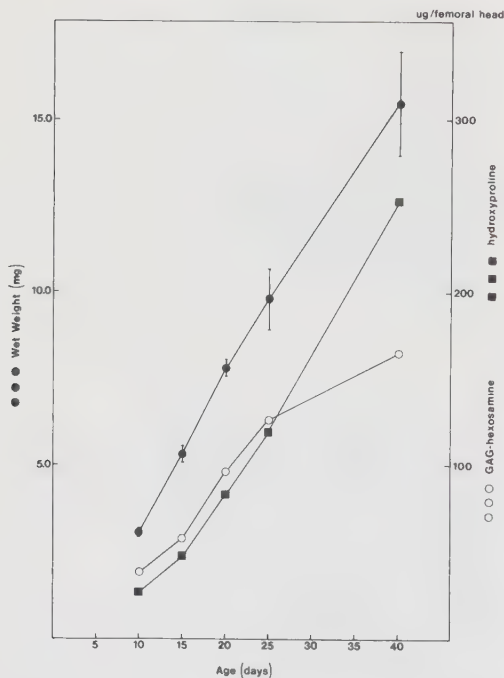


FIG. 2

A diagram showing the wet weight of the femoral head as a function of increased age of the animals. The amounts of glycosaminoglycan-hexosamine and of hydroxyproline were determined as described in Materials and Methods.

five days. Between twenty-five and forty days, however, the proteoglycan content increased more slowly (Figs. 2 and 3). The relative proteoglycan content (concentration) measured as glycosaminoglycan-hexosamine changed with age. Just before and after initiation of calcification at Day 21, however, it was comparatively constant when related both to collagen (hydroxyproline) and to wet weight of tissue (Fig. 3). With increasing age (Day 40) consistently lower values were obtained, indicating a lower rate

of proteoglycan synthesis or increased elimination, or both. It is possible that the proteoglycans have a role as a regulator and that the issue concentration and structure of the proteoglycans change during calcification.

Extraction Yields

Proteoglycans, measured as glycosaminoglycans, were extracted in good yields, ranging from 81.8 to 95.1 per cent, from the femoral head cartilage specimens from various age groups (Table I). The extraction yields were higher than 90 per cent except for the specimens from the forty-day-old rats, in which the epiphysis of the femoral head was almost fully calcified and only 81.8 per cent of the proteoglycans was extracted. It is possible that the mineral hinders extraction of the proteoglycans.

The glucosaminoglycan contents of the cartilage samples were low (Table I), corroborating previous data indicating a lower keratan sulphate content of young carti-

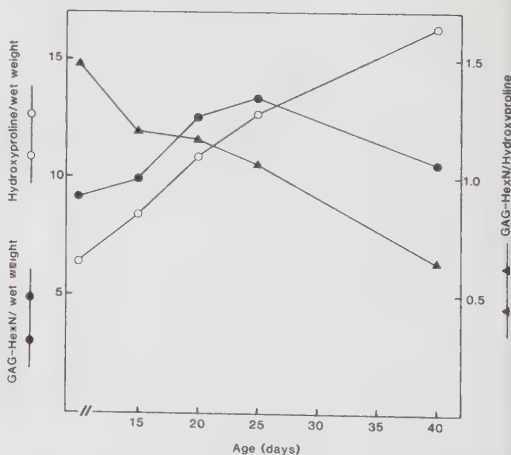


FIG. 3

A diagram showing the relative contents of hydroxyproline and of glycosaminoglycan-hexosamine per femoral head (wet weight). Also shown is the ratio of glycosaminoglycan-hexosamine to hydroxyproline in the femoral head.

TABLE I
COMPOSITION OF EXTRACTS AND RESIDUES FROM FEMORAL HEADS (EXPRESSED AS HEXOSAMINE)

Age (Days)	Extraction Yield*	Glucosaminoglycans/ Galactosaminoglycans ($\mu\text{g}/\mu\text{g}$)		Residue of Glucosaminoglycans/ Hydroxyproline in Tissue ($\mu\text{g}/\mu\text{g}$)
		Extract	Residue	
10	93.1	0.099	0.288	0.058
15	94.8	0.027	0.126	0.017
20	95.1	0.037	0.112	0.017
25	91.2	0.017	0.095	0.021
40	81.8	0.035	0.067	0.018

* Per cent of polysaccharide hexosamine in extract/tissue.

lage¹⁵. The higher glucosaminoglycan content at the age of ten days may reflect a higher cellularity with a higher content of cell-surface-bound heparan sulphate. In support of this, these glucosaminoglycans were not recovered in the A1 fraction (Table II) and therefore probably bound in low-buoyant-density proteoglycans.

The ratio of glucosamine to galactosamine (of glycosaminoglycans) in both the extracts and the residues changed over the age-interval studied (Table I), indicating changes in the composition of matrix glycosaminoglycans. At all times the ratio was lower in the extracts, indicating that a larger proportion of the galactosaminoglycans was extracted.

For the rats that were older than ten days, the amount of hexosamine remaining in the residue after extraction was constant when related to hydroxyproline (Table I). It is possible that these residue proteoglycans are associated with the cells or with the collagen and are not extracted.

Characterization of the A1 Fractions

Proteoglycans were purified by associative cesium chloride density-gradient centrifugation and the A1 fractions were recovered (density, 1.71 grams per milliliter). The hexosamine composition of the A1 fractions showed a higher relative galactosamine content when compared with the entire extract (Table II), indicating that proteoglycans rich in chondroitin sulphate (galactosamine) had a higher buoyant density.

It is noteworthy that the A1 proteoglycans had a much lower relative keratan sulphate content than is usually observed for cartilage proteoglycans⁷. Our animals were,

however, younger than those most often studied, and it is known that the relative keratan sulphate content is lowest in young animals¹⁵. The keratan sulphate content of the A1 fractions from the femoral head consistently increased with increasing age of the animal (Table II), both when related to protein and relative to chondroitin sulphate. The relative chondroitin sulphate content of the proteoglycans, measured as the ratio of galactosamine to protein, varied for the ages studied, indicating structural differences (Table II). The amino-acid composition of the A1 fractions, containing proteoglycans and link proteins, from the rats of various ages showed small differences. The A1 fractions from the ten-day-old rats, however, had higher contents of serine and glutamic acid (glutamine), while the A1 fractions from the forty-day-old rats had somewhat lower glycine values (Table III). The amino-acid composition was similar to that previously described for cartilage proteoglycans⁷, although the glutamic acid contents were somewhat higher in the rat proteoglycans, probably due to the co-analysis of link proteins representing some 10 to 15 per cent of the protein of the A1 fractions.

The ratio of proteoglycan aggregates to monomers in the A1 fractions was determined by Sepharose CL2B column chromatography (Fig. 4). The proteoglycan monomers that are capable of interacting with hyaluronic acid are eluted in the void volume, the aggregate peak, while all other monomers are eluted in the column. The minor component eluted in the total volume may represent the small cartilage proteoglycans recently described¹². The proportion of aggregating proteoglycans was related to the amount of large proteoglycans — the proteoglycans

TABLE II
COMPOSITION OF PROTEOGLYCANS (A1 FRACTION) ISOLATED FROM FEMORAL HEADS

Age (Days)	Glucosaminoglycans*/ Galactosaminoglycans ($\mu\text{g}/\mu\text{g}$)	Galactosaminoglycans*/ Protein† ($\mu\text{g}/\mu\text{g}$)	Glucosaminoglycans*/ Protein† ($\mu\text{g}/\mu\text{g}$)
10	0.0165	1.152	0.019
15	0.0173	2.429	0.042
20	0.0205	1.999	0.041
25	0.0216	2.126	0.046
40	0.0320	1.846	0.059

* Expressed as hexosamine.

† From amino-acid analysis.

TABLE III

AMINO-ACID COMPOSITION OF A1 FRACTIONS FROM FEMORAL HEADS

	Age				
	10 Days	15 Days	20 Days	25 Days	40 Days
Asx	91	92	94	99	97
Thr	60	68	69	69	72
Ser	147	122	121	120	126
Glx	198	169	185	178	180
Pro	56	62	71	65	70
Gly	103	112	93	100	85
Ala	49	45	51	45	48
½ cys	1	1	0	1	0
Val	47	49	52	50	50
Met	0	0	0	0	0
Ile	30	46	30	31	32
Leu	65	89	70	71	72
Tyr	23	16	23	26	25
Phe	24	26	27	33	32
His	21	18	24	26	22
Lys	39	35	39	36	37
Arg	48	48	51	53	52

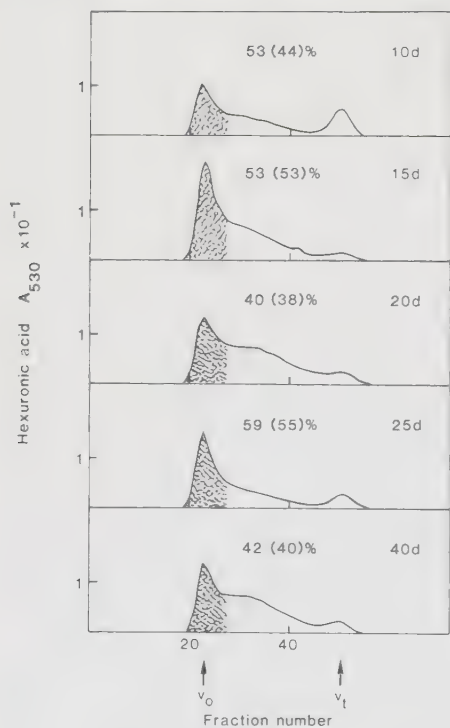


FIG. 4

Sepharose CL2B column chromatography of A1 fractions from the age groups indicated, to determine the proportion of aggregating and non-aggregating proteoglycan monomers. Before chromatography the samples were incubated with 1 per cent high-molecular-weight hyaluronic acid (weight per weight, rooster comb) for twelve hours at 4 degrees Celsius to ensure complete aggregation. The column (0.3 by 140 centimeters) was eluted at a flow rate of 900 microliters per hour. Fractions of 300 microliters were collected and analyzed for content of uronic acid. The hatched area represents the aggregating proteoglycans. Values indicate the relative proportion of the aggregating proteoglycans of the large proteoglycans and, in parentheses, of the total proteoglycans (for details, see text).

in the void volume plus the included non-aggregating large proteoglycans — and to the total amount of proteoglycans. Since the small proteoglycans probably represent another population, only the values for the large proteoglycans will be discussed. The proportion of aggregates is similar in the A1 fractions obtained from the ten and fifteen-day-old animals. In contrast, the sample from Day 20 contained a lower proportion of aggregating proteoglycans (Fig. 4), while on Day 25 the relative content of aggregating monomers was higher and was similar to that of the younger cartilages. At Day 40, the proportion of non-aggregating monomers again was higher. Therefore, both the onset of calcification and the onset of ossification were preceded by a decrease in the proportion of aggregating proteoglycans.

The size distribution of the proteoglycan monomers was determined by chromatography on Sepharose CL2B after the A1 samples were reduced and alkylated (Fig. 5). Such a treatment destroys the tertiary structure of the hyaluronic-acid binding region, thereby preventing the

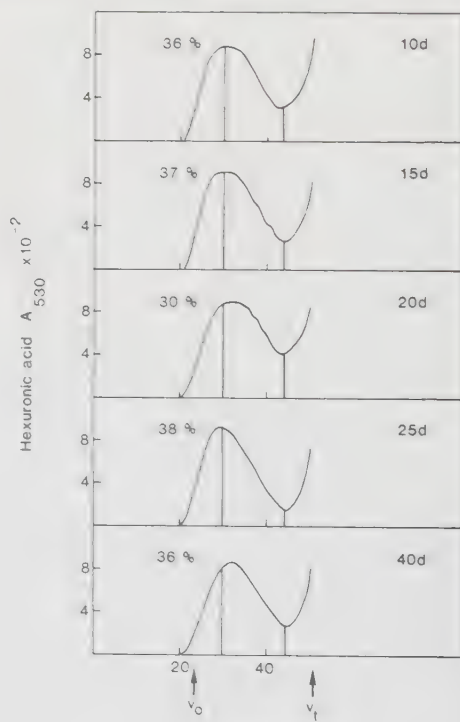


FIG. 5

Sepharose CL2B column chromatography of reduced and alkylated A1 fractions from the age groups indicated, to determine the size of the proteoglycan monomers. The column (0.3 by 140 centimeters) was eluted with 0.5-molar sodium acetate, pH 7.0, at a constant flow rate of 900 microliters per hour. Fractions of 300 microliters were collected and analyzed for content of uronic acid. The total volume peak represents guanidine-hydrochloric acid and reducing agent. The numbers indicate the proportion of the peak eluting in the portion to the left of the marker line.

monomers from interacting with hyaluronic acid. The elution profiles were very similar, although the average size of the monomers was somewhat smaller at Day 20 and Day 40, as indicated by the numbers in Figure 5, when the contents of non-aggregating proteoglycans were higher.

Chromatography on Sepharose CL6B and Sephacryl S-200 of trypsin and papain-digested proteoglycans, respectively, showed that the distribution of chondroitin sulphate chains in clusters along the protein core¹⁰ and the

they were of similar size and had the capability to interact with hyaluronic acid. The ratio of keratan sulphate (glycosaminoglycan-glucosamine) to protein of the proteoglycans from the other age groups remained rather constant, independent of age, although the variable content of chondroitin sulphate (glycosaminoglycan-galactosamine) indicates structural differences. Notably, the proteoglycans from cartilage at Day 15 had the highest content of chondroitin sulphate. Concomitant with the increased pro-

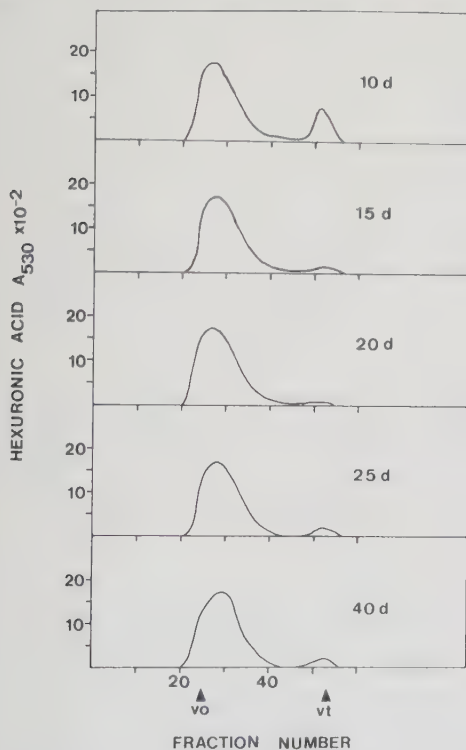


FIG. 6

Sepharose CL6B column chromatography of trypsin digests (see Chemical Methods) of proteoglycans (A1 fraction) from the age groups indicated, to determine the size distribution of chondroitin sulphate clusters. The column (0.3 by 140 centimeters) was eluted with 0.5-molar sodium acetate, pH 7.0, at a constant flow rate of 900 microliters per hour. Fractions of 300 microliters were collected and analyzed for content of uronic acid.

size distribution of these chains in the proteoglycans was the same for all age groups (Figs. 6 and 7). The appearance of carbazole-positive material in the total volume following trypsin digestion (Fig. 6) and papain digestion (Fig. 7) of the various A1 fractions may represent oligosaccharides recently identified in cartilage proteoglycans^{5,23}.

In summary, the proteoglycans from the ten-day-old rat contained more protein and less keratan sulphate than did the proteoglycans from the other age groups. The proteoglycans probably differed structurally, although

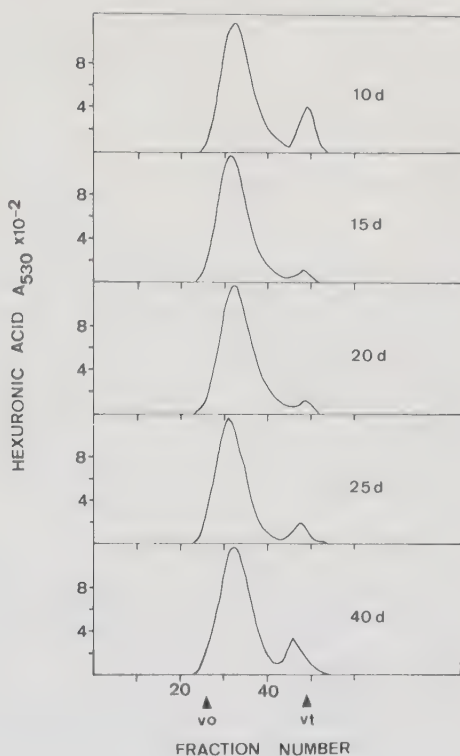


FIG. 7

Sephacryl S-200 column chromatography of papain digests of the proteoglycans (A1 fractions) from the age groups indicated, to determine the size of the chondroitin sulphate chains. The column (0.3 by 140 centimeters) was eluted with 0.5-molar sodium acetate, pH 7.0, at a constant flow rate of 900 microliters per hour. Fractions of 300 microliters were collected and analyzed for content of uronic acid.

portion of non-aggregating proteoglycans at Day 20, the protein content of the molecules increased somewhat (Table II). It has previously been shown that bovine nasal cartilage contains a population of non-aggregating proteoglycans that have a higher protein content compared with the aggregating molecules¹¹. Whether the changes of the proteoglycan structure at the time of onset of calcification can be attributed to breakdown or to altered synthesis cannot now be determined. At the time before ossification at Day 40, again the proteoglycans contained more protein and had a higher proportion of non-aggregating molecules.

Discussion

The present investigation was conducted to characterize the proteoglycans before and during provisional calcification of the cartilage. The femoral head epiphysis of the rat was used because it has the advantage of allowing preparation of samples of calcifying cartilage containing no blood vessels or bone^{16,17}. The first part of the study was a histological control of the samples used, which gave results consistent with those of Koshino¹⁶.

One of the major problems when studying proteoglycan structure is the extreme sensitivity of the molecules to proteolytic degradation. The femoral head epiphysis of the young rat contains neither blood vessels nor bone marrow. Interference from proteases derived from blood cells is therefore minimized. The cartilage of the femoral head epiphysis of the immature rat, then, is very well suited for characterization of macromolecules like the proteoglycans.

The data presented indicate that the proteoglycan structure changes prior to calcification. The relative amount of aggregating proteoglycans decreases, only to again be higher during progressing calcification. The concentration of proteoglycans in the femoral head decreases after the calcification has started and during progression of calcification of the tissue. The changes in proteoglycan content and structure were probably underestimated in the present study.

The femoral head contains two types of cartilage: the epiphyseal cartilage that is changing during provisional calcification, and the articular cartilage. The

amount of proteoglycans in the latter probably increases with age, because of growth, but with no accompanying changes of proteoglycan structure as has been discussed. Therefore, with increasing age the femoral head contains a progressively larger proportion of constant articular-cartilage proteoglycans, masking the alterations of the epiphyseal cartilage molecules.

Pita et al. noted that at the time of calcification the relative content of proteoglycan aggregates as well as the size of such aggregates decreased in interstitial fluid that had been aspirated from epiphyseal tissue. The results presented here support and go beyond their observation, as we showed that the amount of proteoglycans and their composition also change at the time of calcification. Whether this is due to increased degradation or to altered synthesis of the macromolecules is not known at present.

Reddi et al.²¹, in contrast, observed no significant changes in the proteoglycan structure using another model. They induced endochondral ossification by implanting dry bone powder subcutaneously in the rat. It is possible that the events occurring in that model are more complex. It may be that cartilage growth and calcification occur at such rates that changes caused by one process will mask changes caused by others.

There are potentially several ways that proteoglycans can influence the calcification of cartilage. It is possible that they prevent nucleation and crystal growth. Such effects may occur either by exclusion or by ion-exchange effects. In both events, a reduction of the proteoglycan concentration will gradually increase the inhibition.

References

1. ANTONOPOULOS, C. A.; GARDELL, S.; SZIRMAI, J. A.; and DE TYSSONCK, E. R.: Determination of Glycosaminoglycans (Mucopolysaccharides) from Tissues on the Microgram Level. *Biochim. Biophys. Acta*, **83**: 1-19, 1964.
2. AXELSSON, INGE, and HEINEGÅRD, DICK: Fractionation of Proteoglycans from Bovine Corneal Stroma. *Biochem. J.*, **145**: 491-500, 1975.
3. BAYLISS, M. T., and ALI, S. Y.: Age-Related Changes in the Composition and Structure of Human Articular-Cartilage Proteoglycans. *Biochem. J.*, **176**: 683-693, 1978.
4. BLUMENTHAL, N. C.; POSNER, A. S.; SILVERMAN, L. D.; and ROSENBERG, L. C.: Effect of Proteoglycans on In Vitro Hydroxyapatite Formation. *Calcif. Tissue Internat.*, **27**: 75-82, 1979.
5. DE LUCA, S.; LOHMÄNDER, STEFAN; NILSSON, B.; and HASCALL, V. C.: Proteoglycans from Chick Limb Bud Chondrocyte Cultures. Keratan Sulfate and Oligosaccharide which Contain Mannose and Sialic Acid. *J. Biol. Chem.*, **225**: 6077-6083, 1980.
6. ENGFELDT, B., and HJERTQVIST, S.-O.: Studies of the Epiphyseal Growth Zone. I. The Preservation of Acid Glycosaminoglycans in Tissues in Some Histochemical Procedures for Electron Microscopy. *Virchows Arch. Cell. Pathol.*, **1**: 222-229, 1968.
7. HASCALL, V. C., and HEINEGÅRD, D.: Structure of Cartilage Proteoglycans. In *Glycoconjugate Research. Proceedings of the Fourth International Symposium on Glycoconjugates*, edited by J. D. Gregory and R. W. Jeanloz. Vol. 1, pp. 341-374. New York, Academic Press, 1979.
8. HEINEGÅRD, DICK: Polydispersity of Cartilage Proteoglycans. Structural Variation with Size and Buoyant Density of the Molecules. *J. Biol. Chem.*, **252**: 1980-1989, 1977.
9. HEINEGÅRD, D.: Automated Procedures for Determination of Protein, Hexose and Uronic Acid in Column Effluents. *Chem. Scr.*, **4**: 199-201, 1979.
10. HEINEGÅRD, DICK, and HASCALL, V. C.: Characterization of Chondroitin Sulfate Isolated from Trypsin-Chymotrypsin Digests of Cartilage Proteoglycans. *Arch. Biochem. and Biophys.*, **165**: 427-441, 1974.
11. HEINEGÅRD, DICK, and HASCALL, V. C.: Characteristics of the Nonaggregating Proteoglycans Isolated from Bovine Nasal Cartilage. *J. Biol. Chem.*, **254**: 927-934, 1979.
12. HEINEGÅRD, DICK; PAULSSON, MATS; INEROT, SVEN; and CARLSTROM, CHRISTIAN: A Novel Low-Molecular-Weight Chondroitin Sulfate Proteoglycan Isolated from Cartilage. *Biochem. J.*, **197**: 355-366, 1981.
13. HOWELL, D. S.; PITA, J. C.; MARQUES, J. F.; and GATTER, R. A.: Demonstration of Macromolecular Inhibitor(s) of Calcification and Nucleational Factor(s) in Fluid from Calcifying Sites in Cartilage. *J. Clin. Invest.*, **48**: 630-641, 1969.
14. INEROT, S., and HEINEGÅRD, D.: Bovine Tracheal Cartilage Proteoglycans; Variations in Structure and Composition with Age. Unpublished data.
15. INEROT, SVEN; HEINEGÅRD, DICK; AUDELL, LARS; and OLSSON, S.-E.: Articular-Cartilage Proteoglycans in Aging and Osteoarthritis. *Biochem. J.*, **169**: 143-156, 1978.
16. KOSHINO, TOMIHIRO: Development of Femoral Head of the Rat from Calcification to Ossification. *Acta Radiol., Supplementum* 344, pp. 33-46, 1975.
17. KOSHINO, TOMIHIRO, and OLSSON, S.-E.: Normal and Estradiol Induced Calcification of the Femoral Head in Rats. *Acta Radiol., Supplementum* 344, pp. 46-52, 1975.
18. OEGEMA, T. R., JR.; HASCALL, V. C.; and DZIEWIATKOWSKI, D. D.: Isolation and Characterization of Proteoglycans from the Swarm Rat Chondrosarcoma. *J. Biol. Chem.*, **250**: 6151-6159, 1975.
19. PITA, J. C.; MULLER, F.; and HOWELL, D. S.: Disaggregation of Proteoglycan Aggregate during Endochondral Calcification: Physiological Role of Cartilage Lysozyme. In *Dynamics of Connective Tissue Macromolecules*, pp. 247-258. Edited by P. M. C. Burleigh and A. R. Poole.

- Amsterdam, North-Holland, 1975.
20. REDDI, A. H., and HUGGINS, CHARLES: Biochemical Sequences in the Transformation of Normal Fibroblasts in Adolescent Rats. *Proc. Nat. Acad. Sci.*, **69**: 1601-1605, 1972.
21. REDDI, A. H.; HASCALL, V. C.; and HASCALL, G. K.: Changes in Proteoglycan Types during Matrix-Induced Cartilage and Bone Development. *J. Biol. Chem.*, **253**: 2429-2436, 1978.
22. STEGEMANN, HERMANN, and STADLER, KARLHEINZ: Determination of Hydroxyproline. *Clin. Chim. Acta*, **18**: 267-273, 1967.
23. THONAR, E. J.-M. A., and SWEET, M. B. E.: An Oligosaccharide Component in Proteoglycans of Articular Cartilage. *Biochim. Biophys. Acta*, **584**: 353-357, 1979.
-

IN VIVO METABOLISM OF ^{35}S -SULPHATE LABELLED PROTEOGLYCANS IN EPIPHYSEAL,
ARTICULAR AND NASAL CARTILAGES OF GROWING RATS.

Ahnders Franzén and Dick Heinegård

From the Departments of Physiological Chemistry and Stomatognathic Physiology, University of Lund, Sweden¹⁾

1) Send correspondence under adress:
Department of Physiological Chemistry,
P.O. Box 750, S-220 07 Lund 7
Sweden

ABSTRACT.

The biosynthesis and catabolism of proteoglycans were monitored in the epiphyseal and articular cartilages of the femoral head of growing rats as well as in their nasal cartilage. The rats were injected with ^{35}S -sulphate and the amounts of labelled macromolecules in the cartilages were determined at various times after labelling.

The biosynthetic rate of the proteoglycans was rather constant both in epiphyseal and articular cartilages until 2-3 days prior to calcification of the epiphysis, when the rate decreased and most markedly in the epiphyseal cartilage. At this time the elimination rate of the ^{35}S -proteoglycans was much higher in the epiphyseal cartilage than both in the articular and nasal cartilages, where the elimination rate was very low. At the time of the onset of calcification in the epiphyses, the elimination rate of the articular cartilage proteoglycans increased somewhat, but still was lower than that in the epiphyseal cartilage.

With time after labelling the ^{35}S -proteoglycans in the epiphyseal cartilage sedimented gradually slower upon zonal rate centrifugation and contained increasing proportions of proteoglycans with higher electrophoretic mobility. Furthermore the proportion of the labelled molecules that could interact with hyaluronic acid gradually decreased. In contrast the ^{35}S -proteoglycans of the articular cartilage showed only minor changes with time after labelling.

The data indicate that the amounts of proteoglycans in the femoral head epiphyseal cartilage decrease prior to calcification, as a result both of increased degradation of the proteoglycans and a concomitant decreased synthesis of new proteoglycans. Changes also occur in the articular cartilage but only after the onset of calcification and to a smaller degree.

INTRODUCTION.

Most of the bones in the skeleton are formed during fetal life by enchondral ossification of a cartilage skeleton (for review see Bloom and Fawcett, 1975). The central portion of the tubular bones of the skeleton, i.e. the diaphyses, are ossified before birth, the so called primary ossification. One often studied such a bone is the femur, where the femoral head typically remains cartilaginous throughout fetal life with no substructures identifiable by light microscopy (Koshino, 1975). Postnatally, the central portion of the femoral head differentiates to obtain first the typical appearance of an epiphyseal cartilage and then bone, while the peripheral portion differentiates to form the articular cartilage, which remains non-calcified throughout life. Koshino (1975) studied this process, i.e. the secondary ossification in rat femoral head. He observed that the cartilaginous matrix calcified centrally at three weeks after birth. The area of calcification then expanded for another three weeks until the whole proximal epiphysis was calcified, sparing only the articular cartilage.

A central question is what is the nature of the process that initiates calcification and that appears to be unique to the epiphyseal cartilage. A major component of cartilage, that has been considered to be involved in the initiation of the calcification process is the proteoglycans, in particular the aggregates (for review see Buckwalter, 1983). It has been shown that the amount of such proteoglycan aggregates, as well as the amount of aggregating proteoglycans in the epiphyseal cartilage decreases just prior to the onset of calcification (Pita et al., 1970; Franzén et al., 1982 a). Therefore it is likely that these molecules are involved in the process. It is not known, however, whether or not the decreased aggregation is unique to epiphyseal cartilage. Neither is it known if the molecular changes result from an increased degradation of proteoglycans or result from synthesis of a proteoglycan unable to form aggregates. The present investigation was therefore initiated to disclose changes in the metabolism of the proteoglycans in the epiphyseal cartilages before and with ongoing calcification, and also to compare the metabolism of proteoglycans in epiphyseal cartilage with that in the noncalcifying articular and nasal cartilage, respectively.

MATERIAL AND METHODS.

Carrier free ^{35}S -sulphate and ^3H -leucine were obtained from The Radiochemical Centre, Amersham, U.K., agarose (SeaKem ME) was a product from FMC Corp. Marine Colloids Div., Rockland, USA. All other chemicals were of analytical grade.

Animals.

Rats of Wistar strain were bred in our own animal house. Immediately after birth the number of animals in each litter was adjusted to ten, mainly to standardize the growth rate and the size of the animals among the various litters. Only female rats were used in the study.

Labelling of proteoglycans with isotope.

Thirteen-day-old female rats (5-6) from the same litter were kept in a separate cage for 4 hours before they were injected intra-peritoneally with ^{35}S -sulphate (20 uCi/g body weight) in 100 ul of 0.9% sterile sodium chloride. Twelve hours after the isotope injection, the animals were injected intra-peritoneally with 5 mg of sodium sulphate in 250 ul of 0.9% sterile sodium chloride. To minimize contamination of the mother with isotope, the injected animals were kept in a separate cage during labelling and for another 8 h. Other rats, also from one litter, were given isotope (20 uCi/g body weight) at day 12, 17, 18, 19, 20, 21 and 24, respectively. These animals did not receive the nonradioactive sodium sulphate, but were killed 24 h after receiving the isotope.

Tissue preparation.

Animals were sacrificed, by cervical dislocation, at regular time intervals 2, 4, 6, 8, 12 and 17 days after the isotope injection i.e. at ages of 15, 17, 19, 21, 25 and 30 days. The two femoral heads and the nasal cartilage specimen were dissected free from surrounding tissues and transferred to a humidified chamber. The epiphyseal cartilage was separated from the structurally different articular cartilage by microdissection. Immediately after dissection, the wet weights of the different cartilage specimens were measured. The samples were kept at -60°C until further processed.

Extraction of proteoglycans.

The different cartilage samples (2-4 mg of wet weight) were suspended in 500 μ l of 4 M guanidinium chloride, 0.05 M sodium acetate pH 5.8 containing the proteinase inhibitors 5mM benzamidinium chloride, 0.1 M 6-amino-hexanoic acid, 10 mM EDTA (Oegema et al., 1975) and 4 mM N-ethylmaleimide (Heinegård et al., 1981). After extraction for 24 hours at 4°C the mixture was centrifuged for 15 minutes at 10,000 g. The pelleted residue was rinsed twice with 500 μ l of pre-cooled extraction medium and centrifuged as described above. The three supernatants were pooled to form the extract. The residues were suspended in 500 μ l of 1 M sodium hydroxide and dissolved by incubation at 60°C for 12 hrs in sealed tubes. The samples were then neutralized with 10 M acetic acid.

Determination of ^{35}S -sulphate in blood and cartilages.

Blood samples were withdrawn at the time when the animals were killed and weighed samples were dissolved in 500 μ l of 1 M sodium hydroxide as described above and neutralized. The volume was adjusted to 1 ml and the radioactivity was determined with a liquid scintillation counter after the addition of 10 ml of Instagel (Packard Instruments). Weighed samples of the cartilage extracts and solubilized residues, respectively, were precipitated with 10 volumes of ethanol (Paulsson et al., 1983) in the presence of 100 μ g of chondroitin sulphate as a carrier. After incubation overnight at 4°C, the precipitates were recovered by centrifugation for 15 min at 10,000 g and lyophilized. The precipitates were dissolved in 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8, and the radioactivity was determined as described above. Extraction yields of ^{35}S -sulphate proteoglycans were calculated from the ^{35}S -radioactivity present in ethanol precipitates of corresponding extracts and residues.

Agarose-polyacrylamide gel electrophoresis.

Cartilage proteoglycan monomers were separated by electrophoresis on slab gels of agarose-polyacrylamide gels essentially as described by McDevitt & Muir (1971). Minor modifications were, however, introduced to prevent the formation of aggregates, i.e. essentially by dissolving samples in 1 % SDS prior to electrophoresis (procedure to be published). Radioactive components were detected and quantified by fluorography (Chamberlain, (1979).

The gels were immersed in 1 M sodium salicylate, pH 7, dried and the radioactivity was recorded with pre-exposed Kodak X-omat film. The negatives were scanned with a Zenieh soft laser scanner (Biomed. Instr., Chicago, ILL, USA).

Proteoglycan aggregatability.

Extracts of epiphyseal and articular cartilages, containing less than 25 ug of proteoglycan, were mixed with 10 ug of hyaluronate (Healon, a gift from Pharmacia AB, Uppsala, Sweden) and precipitated with ethanol as described above. In preliminary experiments the effect of ethanol precipitation upon proteoglycan aggregability was investigated. No difference in the amount of proteoglycan aggregates formed with added hyaluronate was observed when the aggregation was induced either by dialysis into buffer with low ionic strength or when the proteoglycans plus hyaluronate were precipitated with ethanol prior to electrophoresis. The precipitates were dissolved in 25 ul of 0.01 M Tris-HCl, pH 6.8, containing 5 mM sodium sulphate and proteinase inhibitors, i.e. 5 mM benzamidine chloride, 0.1 M 6-aminohexanoic acid, 0.1 M disodium EDTA and 4 mM N-ethylmaleimide and incubated for 15 hours at 4°C. An equal volume of a 0.02% bromphenol blue solution in 60% (w/v) sucrose and double concentrated electrophoresis buffer, i.e. 0.02 M Tris-acetate pH 6.8 containing 10 mM sodium sulphate was added to the samples, which were electrophoresed on agarose/polyacrylamide gels essentially according to McDevitt & Muir (1971).

Zonal rate centrifugation of proteoglycan monomers.

Samples of the various cartilage extracts were mixed with 200 ug of bovine nasal cartilage proteoglycan monomer (A1-D1) as the carrier. In addition a sample of a solution of ^3H -leucine labelled proteoglycans was added as an internal standard for the sedimentation rate. These ^3H -labelled proteoglycans were prepared from cultures of articular cartilage chondrocytes as described elsewhere (Sommarin & Heinegård, 1983). The mixture of molecules were precipitated with ethanol as described above and the dried precipitates were dissolved in 125 ul of 6M urea, 10 mM Tris-HCl, pH 7. The samples were centrifuged in 4 ml isokinetic gradients of sucrose (10-30 % (w/v) in 6 M urea - 10 mM Tris-HCl, pH 7. After centrifugation for 7 h at 55,000 rpm (311,000 g) and 21°C using an SW 60 Beckman titanium swing-out rotor, the tubes were emptied as described elsewhere (Franzén et al., 1982).

b), fractions of 200 μ l being collected in scintillation vials. The radioactivity was determined as described above.

RESULTS AND DISCUSSION.

To permit monitoring of the molecular changes that occur in the epiphyseal cartilage during the calcification and to compare their nature with those changes occurring in the articular cartilage of the same femoral head a technique was developed for the separation of the two tissue components by microdissection, Fig. 1a. The epiphyseal cartilage could easily be removed with the sharp tip of a canula. Histological studies of the pieces produced showed that the two types of cartilage were well separated (data not shown). Furthermore, it was shown that the growth of both the epiphyseal and the articular cartilage were linear with age, Fig. 1b, as would be expected from previous studies (Franzén et al., 1982 a).

In vivo labelling of proteoglycans.

The amounts of free isotope in the circulation and in the cartilages were checked at the times indicated in Fig. 2, insert. Its concentration in the blood was found to be negligible at 2 days after the isotope injection, showing that the chase had been efficient. Furthermore, no free isotope could be detected in cartilage extracts from this time. Therefore it can be assumed that the changes in amounts of ^{35}S -labelled proteoglycans seen at 15 days of age and later (3 days or more after labelling) only result from catabolism. A further control is that cartilage from the control male littermates who were not given isotope, contained no labelled proteoglycans showing the lack of transfer of isotope from their mother.

Catabolism of ^{35}S -sulphate labelled proteoglycans.

The turnover of labelled proteoglycans was highest in the epiphyseal cartilage. Indeed already at 17 days of age, i.e. 5 days after labelling, contents of labelled proteoglycans had decreased to 80 %, but only in the epiphyseal cartilage and the contents continued to decrease with a half life of about 9.5 days, until day 25 when most of the cartilage had become

hypertrophic and calcification had started, Fig. 2. Until day 21 of age, no decrease in the contents of labelled proteoglycans was observed neither in the articular cartilage of the same femoral head nor in the nasal cartilage of the animal indicating a very slow turnover. After the onset of calcification in the femoral head at day 21, however, the content of labelled proteoglycans gradually decreased most markedly in the articular cartilage, but also to some extent in the nasal cartilage. It appears that more generalized changes in the elimination of cartilage proteoglycans occurs at this age. The elimination rate of the proteoglycans in the epiphyseal cartilage, however, is high already before the onset of calcification, coinciding with the decreasing contents of proteoglycans in the cartilage (Franzén et al., 1982a).

Although the extraction yield of proteoglycans was only 50-60 %, it remained constant at all times. Therefore, both those proteoglycans not solubilized and those extracted with guanidinium hydrochloride showed similar catabolism.

In order to characterize the labelled proteoglycans they were electrophoresed on agarose-polyacrylamide gels and subjected to zonal rate centrifugation in sucrose gradients. The electrophoresis patterns, Fig. 3, show that particularly in the epiphyseal cartilage a fraction of proteoglycan monomers with somewhat higher electrophoretic mobility appears with time. The change in the migration pattern of the articular cartilage proteoglycan monomers is less marked, Fig. 3. The sedimentation profiles, Fig. 4, show a progressively slower sedimentation rate with time of the epiphyseal cartilage proteoglycans, while those in articular cartilage did not change with time after labelling. These data, then, indicate that the proteoglycans from the epiphyseal cartilage become smaller with time possibly a result from partial proteolytic cleavage. Further support was obtained by measurements of aggregatability with hyaluronic acid, Fig. 5. Notably, with time, the labelled epiphyseal cartilage proteoglycans contain a gradually decreasing proportion of aggregating proteoglycans. The proteoglycans in the articular cartilage, on the other hand, contained a decreasing proportion of aggregating labelled proteoglycans only after the onset of calcification, i.e. at the time when their elimination rate increased.

Biosynthesis of proteoglycans.

In another set of experiments aimed at studying the biosynthesis of the cartilage proteoglycans the animals were injected with ^{35}S -sulphate and killed 24 h later. The biosynthetic rate of the proteoglycans remains rather constant from 12 days of age until 17 days of age, Fig. 6. At day 18 to 19, however, the synthesis of proteoglycans in the epiphyseal cartilage was much decreased and decreased even further until day 21, at the time of beginning calcification. The synthesis of proteoglycans in the articular cartilage also decreased, although not to the extent of the decrease observed in the epiphyseal cartilage, Fig. 6.

CONCLUDING REMARKS

The present investigation shows that there are major changes in the turnover of proteoglycans during the enchondral ossification. Catabolism of proteoglycans is increased, more pronouncedly so in the epiphyseal cartilage than in other cartilages. This increased catabolism appears to result in a decreased tissue content primarily of aggregating proteoglycans, consistent with results of biochemical studies (Franzén et al., 1982a). Furthermore, also the synthesis of proteoglycans in the epiphyseal cartilage decreases and more so than in the other cartilages studied. As a result the content of proteoglycans, primarily of aggregates, in epiphyseal cartilage decreases drastically at the time of calcification. These findings are strong indications of the involvement of proteoglycans in the regulation of cartilage calcification.

Acknowledgements.

Grants were obtained from the Swedish Medical Research Council (05668), Folksam's Yrkeskadors Stiftelse, Kocks Stiftelser, Österlunds Stiftelse, Konung Gustaf V:s 80-års fond and The Faculties of Medicine and Odontology, University of Lund. Skilful technical assistance by Miss Carina Persson and Miss Sara Axelsson is appreciated.

REFERENCES.

- Bloom, W. and Fawcett, D.W. A Textbook of Histology 10th ed., pp 262-279, Philadelphia, Saunders 1975
- Buckwalter, J.A. (1983) Clin. Orthop. and Rel. Res. No 172, 207-232
- Chamberlain, J.P. (1979) Anal. Biochem. 98, 132-135
- Franzén, A., Björnsson, S. and Heinegård, D. (1982 b) Anal. Biochem. 120, 38-45
- Franzén, A., Heinegård, D., Reiland, S. and Olsson, S.-E. (1982 a) J. of Bone and Joint Surg. Vol. 64-A, 558-566
- Heinegård, D., Paulsson, M., Inerot, S. and Carlström, C. (1981) Biochem. J. 197, 355-366
- Koshino, T. (1975) Acta Radiologica Suppl. 344, 33-46
- McDevitt, C. and Muir, H. (1971) Anal. Biochem. 44, 612-622
- Oegema, T., Hascall, V.C. and Dziewiatkowski, D. (1975) J. Biol. Chem. 250, 6151-6159
- Paulsson, M., Sommarin, Y. and Heinegård, D. (1983) Biochem. J. 212, 659-667
- Pita, J.C., Cuervo, L.A., Madruga, J.E., Müller, F.J. and Howell, D.S. (1970) J. Clin. Invest. 49, 2188-2197
- Sommarin, Y. and Heinegård, D. (1983) Biochem. J. 214, 777-784

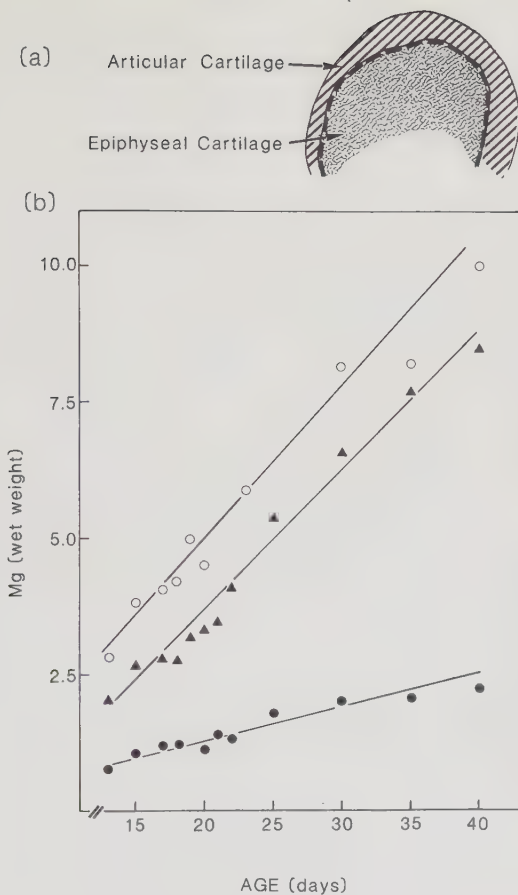


Figure 1.a. Schematic illustration of the dissection of femoral head.

Articular and epiphyseal cartilage samples, respectively, were prepared from growing rat femoral head using the tip of a narrow canula.

b. The wet weight of various cartilages as a function of increased age of female rats.

The wet weight of the whole femoral head (○—○), of the isolated articular (▲—▲) and epiphyseal (●—●) cartilage specimen were determined. Each point in the diagram represents a mean of two animals (the S.E.M. is within the geometry of the dot). Further experimental details are described in Material and Methods.

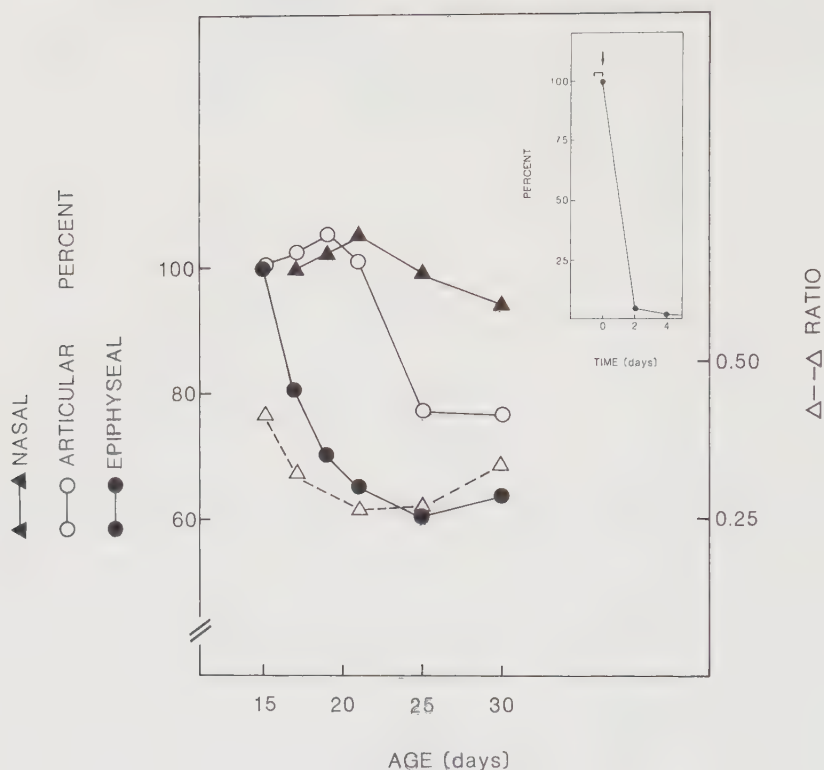


Figure 2. Elimination of ^{35}S -labelled proteoglycans from cartilage.

The amount of labelled proteoglycan in each cartilage at 2 days after isotope injection is used as a reference (100%) value. The figure shows the total amount of labelled macromolecules remaining in epiphyseal (●—●), articular (○—○) and nasal (▲—▲) cartilage relative to that 2 days after the isotope injection. The ratio between the labelled proteoglycans remaining in the epiphyseal cartilage to those remaining in the articular cartilage of the same femoral head is also shown ($\Delta-\Delta$). Insert shows the concentration of isotope in blood samples as ratio to the value immediately after injection of isotope. The arrow indicates the injection of non-radioactive sulphate.

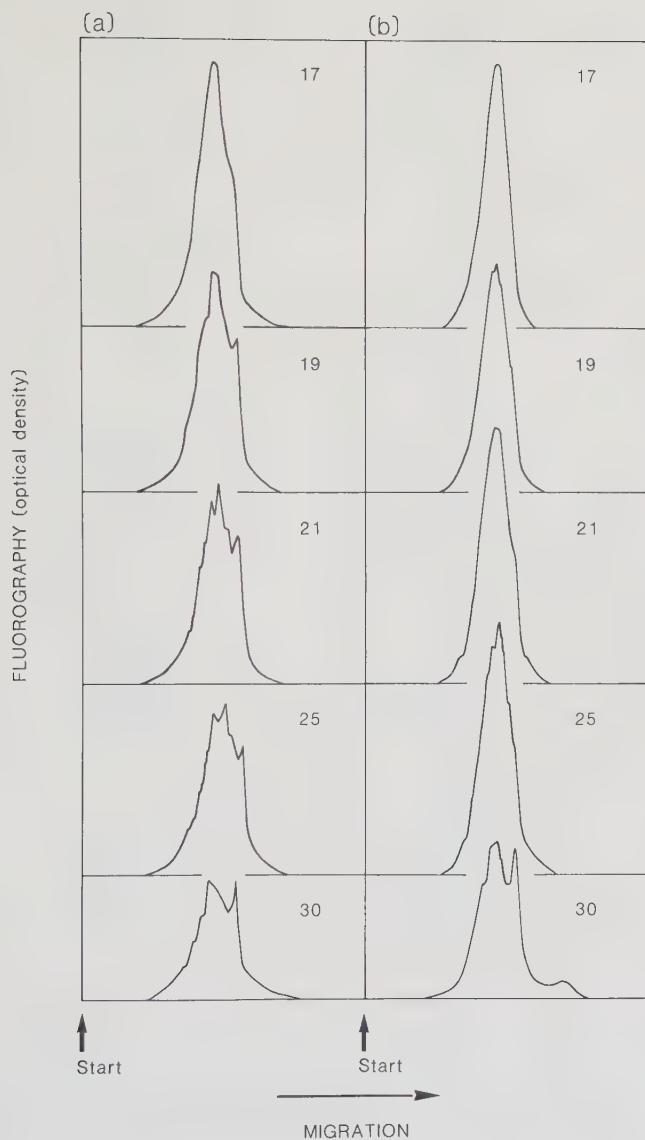


Figure 3. Agarose-polyacrylamide gel electrophoresis of ^{35}S -labelled proteoglycans.

The samples were prepared from epiphyseal (a) and articular (b) cartilage at the ages (days) indicated, after isotope injection at 13 days of age. The radioactivity was detected by fluorography. Further experimental information is given in Material and Methods.

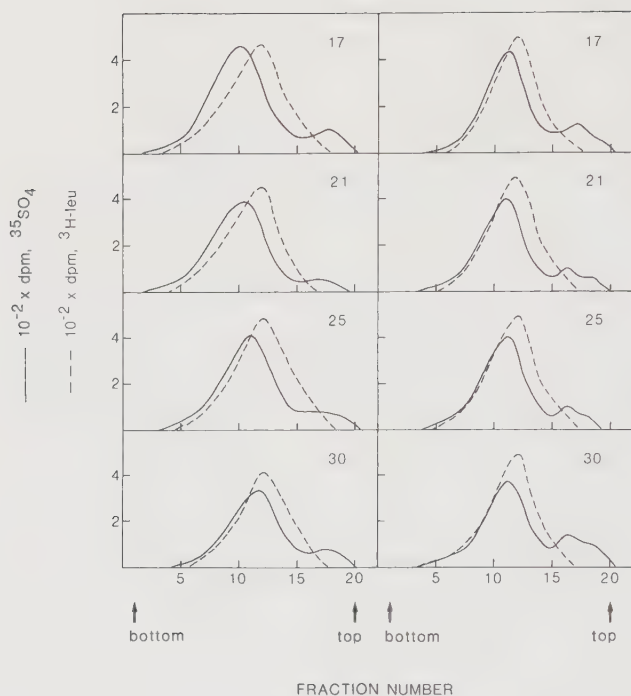


Figure 4. Sucrose density gradient centrifugations of ^{35}S -sulphate proteoglycans.

The samples were prepared from epiphyseal (left) and articular (right) cartilages at the ages (days) indicated after isotope injection at 13 days of age. A preparation of ^3H -leucine labelled proteoglycans was included as reference. The experimental protocol is given in Material and Methods.

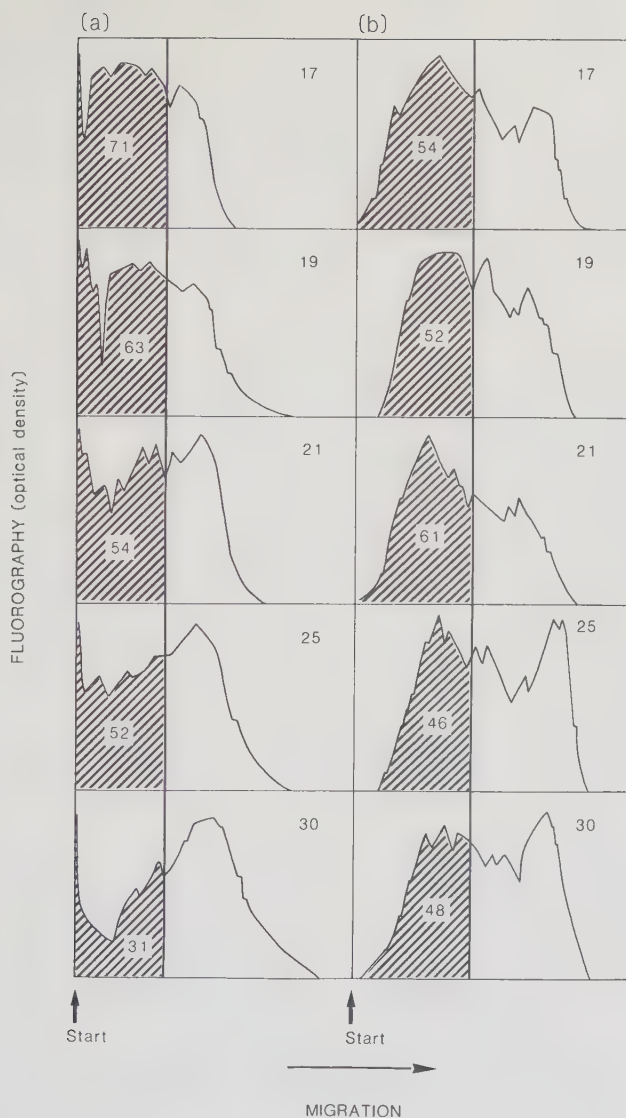


Figure 5. Determination of aggregability with hyaluronic acid by agarose-polyacrylamide gel electrophoresis of ^{35}S -labelled proteoglycans.

The ^{35}S -sulphate labelled proteoglycans were extracted from epiphyseal (a) and articular (b) cartilage, precipitated with ethanol after a large excess of hyaluronate had been added. After incubation of the redissolved precipitate to allow aggregation, the samples were electrophoresed. The content of aggregating proteoglycans (hatched area) in the extracts is expressed as percentage of the total (area). The radioactivity was detected by fluorography and the films were scanned using a densitometer. Further details of the experiment given in Material and Methods.

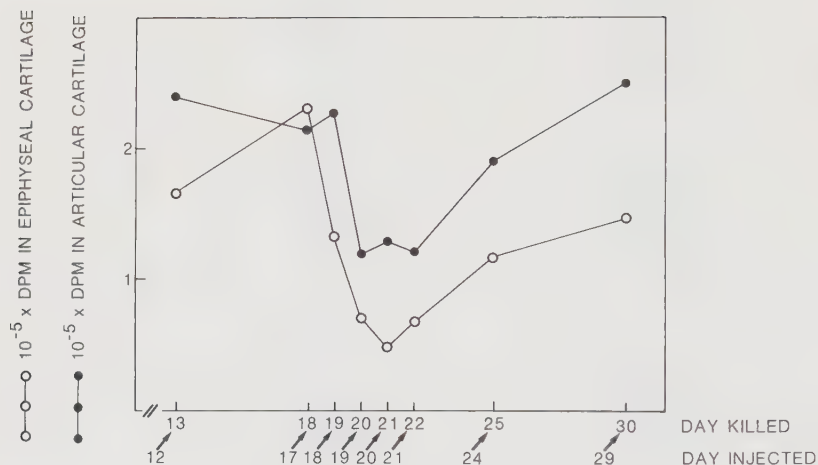


Figure 6. Biosynthesis of ³⁵S-labelled proteoglycans.

Animals were killed 24 hrs after injection of ³⁵S-sulphate and total contents of labelled proteoglycans in femoral head epiphyseal (○—○) and articular (●—●) cartilage were measured. Further technical details are given in the text.

Variations in the composition of bovine hip articular cartilage with distance from the articular surface

Ahnders FRANZÉN, Sven INEROT, Sven-Olof HEJDERUP and Dick HEINEGÅRD

Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden

(Received 15 September 1980/Accepted 17 February 1981)

Punch biopsies of bovine hip articular cartilage was sectioned according to depth and the proteoglycans were isolated. The mid-sections of the cartilage contained more proteoglycans than did either the superficial or the deepest portions of the cartilage. The most superficial 40 μm of the cartilage contained relatively more glucosaminoglycans compared with the remainder of the cartilage. The proteoglycans recovered from the surface 200 μm layer contained less chondroitin sulphate, were smaller and almost all of these molecules were able to interact with hyaluronic acid to form aggregates. From about 200 μm and down to 1040 μm from the surface, the proteoglycans became gradually somewhat smaller, probably owing to decreasing size of the chondroitin sulphate-rich region. The proportion of molecules that were able to interact with the hyaluronic acid was about 90% and remained constant with depth. The proteoglycans from the deepest layer near the cartilage–bone junction contained a large proportion of non-aggregating molecules, and the average size of the proteoglycans was somewhat larger. The alterations in proteoglycan structure observed with increasing depth of the articular cartilage beneath the surface layer (to 200 μm) are of the same nature as those observed with increasing age in full-thickness articular cartilage. The articular-cartilage proteoglycans were smaller and had much higher keratan sulphate and protein contents than did molecules isolated from bovine nasal or tracheal cartilage.

Cartilage has a high elasticity and resilience, largely because of the high content of proteoglycans in the tissue (Kempson *et al.*, 1971; Harris *et al.*, 1972; Scott, 1975; Kempson, 1975). The proteoglycans consist of a central protein core and a large number of charged and extended polysaccharide side chains. The proteoglycans therefore will occupy a very large domain. Several proteoglycans bind to one hyaluronic acid molecule, forming a very large proteoglycan aggregate. Part of the proteoglycan protein core is the hyaluronic acid-binding region, containing little or no polysaccharide. Another portion of the protein core, containing most of the keratan sulphate chains, constitutes the keratan sulphate region, probably interspaced between the hyaluronic acid-binding region and the part containing the chondroitin sulphate chains (Heinegård & Axelsson, 1977; Hascall & Heinegård, 1979).

The quantity of glycosaminoglycans is highest in the mid-portions of the cartilage and lower both towards the surface and towards the osteochondral junction (Stockwell & Scott, 1967; Maroudas *et al.*, 1969, 1973; Lipshitz *et al.*, 1976; Maroudas, 1979). It has also been shown that the glycosaminoglycan

glucosamine/galactosamine ratio increases with increasing depth in the cartilage (Stockwell & Scott, 1967). Such information, when related to the current model of proteoglycan structure, may indicate that the size of the proteoglycans is smaller in the deeper portions of the cartilage.

The present investigation was initiated to study the changes in articular-cartilage proteoglycan structure with distance from the articular surface.

Materials and methods

Chemicals

Aristar-grade HCl was obtained from BDH, Poole, Dorset, U.K. Papain and diphenylcarbamoyl chloride-treated trypsin were from Sigma Chemical Co., St. Louis, MO, U.S.A. Sepharose CL 2B, Sepharose 2B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Tissue-Tec II (CM-cellulose) was obtained from Miles Laboratories, Naperville, IL, U.S.A. The other chemicals used were all of analytical grade. High-molecular-weight rooster comb hyaluronic acid (Healon) was a gift from Pharmacia. This prepara-

tion of hyaluronate chromatographs in the void volume of a Sepharose 2B column.

Tissue preparation

Femoral-head articular cartilages from three different 2-year-old bulls were used. Within 10 min after the death of the animals, their left hip joints were carefully opened and the femoral head was divided from the rest of the femur and removed. The synovial fluid was blotted off the articular surface with soft tissue paper. By using a specially made punch of diameter 20 mm, a cartilage-bone cylinder was prepared from the most weight-bearing area of the cartilage, just dorsal and lateral to ligamentum teres femoris. The specimen was immediately removed from the punch, frozen in liquid N₂ and transported to the laboratory immersed in liquid N₂. The cartilage-bone cylinder was longitudinally divided into small pieces with a cross-sectional area of about 3 mm². The surface of each of these pieces had only a minor curvature and was for practical purposes considered to have no curvature. Each piece was mounted to an aluminium cylinder with the aid of Tissue-Tec and fitted to a freeze-microtome. All operations were done with the tissue pieces frozen at -30°C. Serial sections of 20 µm thickness were prepared from the articular-cartilage surface to the cartilage-bone junction. Each cartilage piece yielded 62 sections. Cartilage from three animals was pooled separately into two sets of seven groups, with distances from the articular surface of 0-40, 40-240, 240-440, 440-640, 640-840, 840-1040 and 1040-1240 µm for animals A and C and 0-40, 40-120, 120-200, 200-280, 280-360, 360-440 and 440-1240 µm for animal B. Special care was taken to ensure that the cartilage specimens did not thaw during preparation and sectioning, to minimize degradation of the proteoglycans.

Histological investigation

A small cartilage-bone cylinder of diameter 2 mm was taken adjacent to the preparative punch biopsy, for histological investigation after staining with hematoxylin/eosin.

Preparation of proteoglycans

Proteoglycans were extracted from each pool of sectioned cartilage from animals A and B with 3 ml of 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, containing the proteinase inhibitors EDTA (0.01 M), benzamidine hydrochloride (0.005 M) and 6-aminoheptanoic acid (0.1 M) (Oegema *et al.*, 1975). Extraction was done for 24 h at 4°C on a shaker. Extracts and residues were separated by filtration through a nylon sieve, which was rinsed twice with 0.5 ml of the 4 M-guanidinium chloride solution used for extraction. The washings were pooled with the original extract and a sample of each pool was

removed for determination of extraction yield. The residues were stored frozen until processed further.

The extracts were dialysed against 10 vol. of 0.05 M-sodium acetate, pH 5.8, containing the proteinase inhibitors described above. The density of the dialysis residue was adjusted to 1.64 g/ml by addition of solid CsCl. The solutions (7 ml/tube) were then centrifuged at 4°C for 60 h in an MSE 65 ultracentrifuge in the 10 × 10 ml titanium angle rotor at 35 000 rev./min (85 000 g, r_{av} 6.44 cm). After termination of the centrifugation, the tubes were frozen in an absolutely vertical position by carefully immersing them in liquid N₂. While still frozen the tubes were cut with a saw in a special guide to give the bottom two-fifths (the A1 fraction) and the top three-fifths (the A2 fraction). The densities of the fractions were determined with a 300 µl constriction pipette as a pycnometer. The fractions were then dialysed against one change of 0.5 M-sodium acetate and then extensively against distilled water, and then freeze-dried.

Agarose/polyacrylamide-gel electrophoresis

Analytical agarose/polyacrylamide-gel electrophoresis of the reduced and alkylated A1 fractions from the different layers was carried out as described by McDevitt & Muir (1971). The gels were scanned by measuring the A_{320} .

Column chromatography

Analytical columns (0.3 cm × 140 cm) of Sepharose CL 2B and Sephacryl S-200 were eluted with 0.5 M-sodium acetate, pH 7.0, at 4°C. Usually 150 µg of proteoglycan or digest thereof was applied. The uronic acid content of each fraction was determined by an automated version of the carbazole method (Heinegård, 1973). Alternatively about 25 µg of proteoglycan was chromatographed on a Sepharose 2B column (0.5 cm × 140 cm), eluted with 0.25 M-Na₂SO₄ at 15°C with a constant-flow infusion pump. The effluent was continuously monitored for A_{206} with a Uvicord S monitor. At this wavelength the absorbance for protein is about 6 times that of glycosaminoglycans on a dry-weight basis.

The ratios of proteoglycan aggregates to monomers in the A1 fractions were determined after chromatography on Sepharose CL 2B. To ensure binding of all proteoglycan monomers capable of interaction, high-molecular-weight hyaluronic acid (1% of proteoglycan dry wt.) was added to the A1 fractions before chromatography (Hascall & Heinegård, 1979).

The size distribution of the proteoglycan monomers was determined by chromatography on Sepharose CL 2B and Sepharose 2B after reduction and alkylation of the A2 preparations (Heinegård, 1977).

Typically 25–150 μg of fraction A1 was dissolved in 150 μl of 4 M-guanidinium chloride/0.05 M-Tris/HCl/0.005 M-dithiothreitol, pH 7.6, and incubated at 37°C for 5 h. Iodoacetic acid (20 μl of a 0.1 M solution) was then added and the sample was incubated for 12 h at room temperature in the dark. Reduction and alkylation prevent aggregate formation by proteoglycan monomers (Heinegård, 1977).

The size distribution of the chondroitin sulphate chains was determined by chromatography on Sephacryl S-200 after digestion of 150 μg of fraction A1 with 1 μl of a suspension of crystalline papain (25 mg/ml) at 65°C for 3 h in 150 μl of 0.005 M-sodium phosphate buffer, pH 7.0, containing 0.005 M-EDTA and 0.005 M-cysteine hydrochloride.

Isolation and determination of glycosaminoglycans

Extraction yields were determined as follows. The residues and a known volume of the extracts from animal A were each dialysed against water and freeze-dried. The samples were then suspended in 500 μl of the buffer described above and digested with 2 μl of the suspension of crystalline papain at 65°C for 15 h. The released glycosaminoglycans were then isolated from the glycopeptide fraction by Ecteola-cellulose ion-exchange chromatography (Axelsson & Heinegård, 1975). Glycosaminoglycans were eluted with 2 M-HCl. Total tissue glycosaminoglycan content and distribution was measured after papain digestion of the sections from animal C with 15 μl of the papain suspension for 72 h at 65°C. Glycosaminoglycans were isolated as described above.

Chemical methods

Hexosamine contents of fractions were determined by using a Durrum automatic amino acid analyser after hydrolysis of samples in 4 M-HCl in sealed tubes under argon at 100°C for 10 h. Hydrolysis for determination of amino acids was with 6 M-HCl at 110°C for 24 h in sealed tubes under argon. The amino acids were then determined by using the amino acid analyser. Hydroxyproline

was determined as described by Stegemann & Stalder (1967).

Results and discussion

Morphology of tissue specimens

The articular cartilage showed no macroscopic sign of degeneration. The histology of the tissue biopsy showed three major regions. The region nearest to the surface extended from 0 to 40 μm and contained fibres and discoidal cells arranged parallel to the articular surface. The second region in the middle of the cartilage extended from 40 to 1040 μm and had an appearance typical for cartilage, with few cells and an abundant intercellular matrix. The region (200 μm) just above the cartilage–bone junction (1040–1240 μm from the cartilage surface) contained spheroidal cells and represents the proliferation area of the cartilage bone junction. The thickness of the articular cartilage measured in the microscope was 1250–1300 μm , in good agreement with the thickness obtained from the sum of the sections prepared for chemical investigation.

Distribution of glycosaminoglycans

Available data indicate that the collagen content of hip articular cartilage is constant at different depths of the cartilage (Lipshitz *et al.*, 1975; Maroudas, 1979). It was therefore assumed that the collagen content was constant and that the ratio of glycosaminoglycan hexosamine to hydroxyproline can be taken to indicate the proteoglycan content of the sections. One set of sections from animal C was digested with papain, and weighed samples from the digests were taken for hydroxyproline and glycosaminoglycan determinations. The higher content of glycosaminoglycans in the mid-sections compared with sections both from the surface and from the osteochondral junction (Table 1) corroborates previous data (Stockwell & Scott, 1967; Maroudas *et al.*, 1969; Lipshitz *et al.*, 1976; Maroudas, 1979). The content of glycosaminoglycan glucosamine, probably indicating keratan sulphate, varied only

Table 1. Glycosaminoglycan contents of sections from articular cartilage from animal C

Glycosaminoglycans are quantified as hexosamine of isolated glycosaminoglycans and collagen as hydroxyproline. Ratios are w/w.

Section	Glycosaminoglycan hexosamine hydroxyproline	Glycosaminoglycan galactosamine hydroxyproline	Glycosaminoglycan glucosamine galactosamine
0–40 μm	0.21	0.14	0.54
40–240 μm	0.34	0.25	0.35
240–440 μm	0.50	0.35	0.43
440–640 μm	0.50	0.36	0.39
640–840 μm	0.49	0.34	0.45
840–1040 μm	0.44	0.30	0.45
1040–1240 μm	0.27	0.18	0.47

Table 2. *Glucosamine/galactosamine ratio of glycosaminoglycan fractions of proteoglycan aggregate (A1) fraction, extract and extraction residue, and glycosaminoglycan extraction yields (animal A)*

Abbreviation: n.d., not determined.

Section	Glucosamine/galactosamine in glycosaminoglycan fraction			Extraction yield (% of total)	Glucosamine/protein in A1 fraction	Galactosamine/protein in A1 fraction
	Extract	Residue	A1 fraction			
0–40 μ m	0.81	0.74	n.d.	98.6	n.d.	n.d.
40–240 μ m	0.25	0.40	0.49	97.4	0.31	0.62
240–440 μ m	0.28	0.57	0.39	99.1	0.36	0.93
440–640 μ m	0.37	0.60	0.46	97.6	0.38	0.82
640–840 μ m	0.33	0.51	0.61	98.8	0.40	0.65
840–1040 μ m	0.41	0.48	0.66	96.3	0.36	0.55
1040–1240 μ m	0.32	0.55	0.48	96.5	0.43	0.89

Table 3. *Amino acid composition of the proteoglycans (A1 fractions) from the various tissue sections (animal A)*

Values are expressed as residues per 1000.

Amino acid	Section ...	40–240 μ m	240–440 μ m	440–640 μ m	640–840 μ m	840–1040 μ m	1040–1240 μ m
Asp		81	78	79	79	81	82
Thr		57	56	54	54	53	57
Ser		154	144	154	156	169	156
Glu		131	146	147	152	136	149
Pro		70	74	71	76	60	74
Gly		150	163	179	169	181	151
Ala		66	62	63	68	57	62
Cys		8	6	4	6	3	2
Val		50	47	44	42	48	51
Met		4	2	2	3	0	0
Ile		30	28	25	24	21	25
Leu		62	63	60	58	60	66
Tyr		19	24	21	21	19	21
Phe		28	30	27	26	22	24
His		21	16	15	15	20	15
Lys		35	35	29	27	40	34
Arg		34	27	26	23	30	31

little, although the surface layer contained relatively more.

Extraction of proteoglycans

Extraction yields (animal A) were much higher (96–99%; Table 2) than those reported previously from other types of cartilage (for references see Hascall & Heinegård, 1979). Possibly the smaller pieces of cartilage obtained by sectioning result in improved extraction.

Corroborating previous results (Inerot *et al.*, 1978), the residues from all tissue samples contained relatively more glucosaminoglycans than did the extracts. The ratio of glucosamine/galactosamine in the glycosaminoglycan fractions (Table 1) was very high in the 0–40 μ m section in both the extract and the residue. It is likely that this layer of the cartilage may contain relatively more keratan sulphate or heparan sulphate. Stockwell & Scott

(1967) showed that the surface layer of human articular cartilage had a higher content of glycosaminoglycan glucosamine, mainly in the sulphated-polysaccharide fraction. The second pool of slices, corresponding to 40–240 μ m depth, showed the lowest ratio. The relative glucosaminoglycan content, however, increased somewhat with further depth of the cartilage.

Composition of A1 fractions

Contents of hexosamines, amino acid and protein of A1 fractions (density 1.71 g/ml) from animal A were determined. The glucosamine/galactosamine ratios were higher than for the corresponding extracts, but still tended to increase with depth of the cartilage. The layer from the cartilage–bone junction, however, again had a lower ratio (Table 2). The relative content of glucosamine was much

higher than that observed from bovine nasal and tracheal cartilage (Hascall & Heinegård, 1974), indicating a higher relative content of keratan sulphate, corroborating data from other articular cartilages (Inerot *et al.*, 1978). The amino acid compositions of the A1 fractions were very similar (Table 3). Protein contents of the fractions were determined as the sum of the amino acids. The ratio of hexosamines to protein was calculated (Table 2). Unfortunately the amount of material recovered from the A1 fraction from the top section was not sufficient for further analysis. The keratan sulphate/protein ratio (determined as glucosamine/protein) increased somewhat with distance from the cartilage surface. The chondroitin sulphate/protein ratio (determined as galactosamine/protein) was comparatively low in the A1 fraction recovered from 40 to 240 μm . The A1 fraction from the section 240–440 μm had a higher relative chondroitin sulphate content than any of the other fractions. The chondroitin sulphate/protein ratio gradually decreased with increasing depth of the cartilage. The sample near the cartilage–bone junction had a higher relative content of galactosamine. In summary, the composition of the A1 fractions recovered from sections at 240–1040 μm showed that the relative contents of chondroitin sulphate decreased, the contents of protein increased and the contents of keratan sulphate remained relatively constant. The decreased chondroitin sulphate content was not due to decreasing size of the chains, as discussed below. The 40–240 μm -section A1 fraction contained the least chondroitin sulphate and probably also less keratan sulphate than the other fractions analysed.

Gel chromatography

The size of the proteoglycan monomers from the various layers from animal A was determined by Sepharose-2B chromatography of the reduced and alkylated A1 fractions. The uronic acid (glycosaminoglycan) tracings of the chromatograms (Fig. 1) showed that the size distribution of the proteoglycans in the top fraction analysed (40–240 μm) differed, in that these molecules were on the average considerably smaller than those of the deeper layers. The proteoglycan monomers recovered from the layer 240–440 μm were on average somewhat larger than any of the others. The tracings indicate that the average size of the proteoglycan monomers decreases (see Table 4), in the samples from 240 and down to 1040 μm from the cartilage surface. All the samples showed approximately the same polydispersity, except for the much less polydisperse sample from the surface layer (40–240 μm). The gel-chromatographic data are consistent with changes in the composition of the proteoglycan

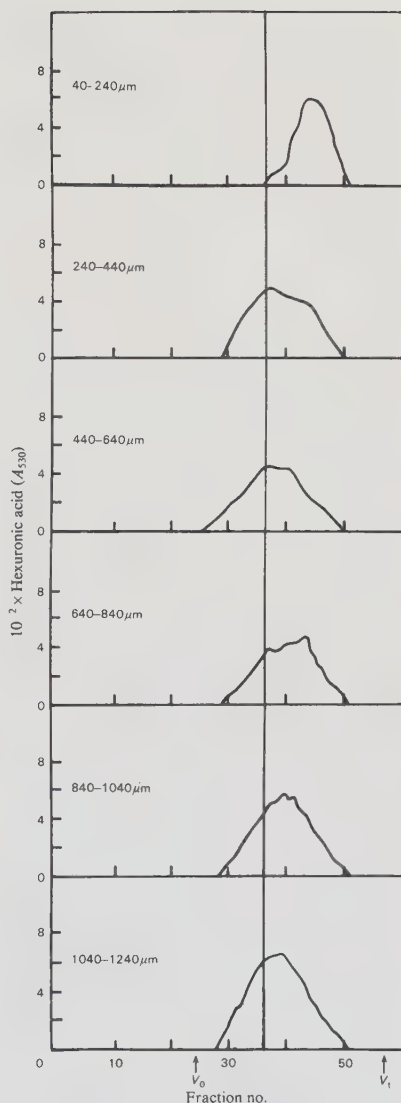


Fig. 1. Sepharose CL 2B chromatograms of proteoglycan monomers (reduced and alkylated A1 fractions from animal A)

The column (0.3 cm $\times$ 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μl fractions. The numbers indicate depth of cartilage layers from the surface. Abbreviations: V_0 , void volume; V_t , total column volume.

Table 4. Relative proportion of proteoglycans in fractions 27–36 and 37–51 in the chromatograms in Fig. 1

Distance from articular surface (μm)	Uronic acid (% of total in chromatogram)	
	Fraction 27–36	Fraction 37–51
40–240	0	100
240–440	32	68
440–640	39	61
640–840	21	79
840–1040	25	75
1040–1240	35	65

monomers discussed above. The top fraction analysed differs markedly, whereas the proteoglycans from the other sections show a tendency to a continuous change in the various parameters analysed. In order to determine the size of the proteoglycan monomers from the surface layer (0–40 μm) and to characterize the change in size of the proteoglycans in the uppermost 440 μm sections, the reduced and alkylated A1 fractions from the layers 0–40, 40–120, 120–200, 200–280, 280–360, 360–440 and 440–1240 μm (animal B) were separately chromatographed on Sepharose 2B. Because of the small amount of sample available, the procedure for analysis had to be altered. Instead of monitoring proteoglycans by uronic acid analysis, their A_{206} was continuously measured in column effluents, as discussed in the Materials and methods section. The chromatograms (Fig. 2) reveal that the proteoglycans in the uppermost 200 μm are distinctly smaller than those of the deeper layers and that the proteoglycans in the most superficial layer are of similar nature to those in the nearest layers.

It was considered that the changing size of the proteoglycan monomer may be due to changes in the size of the chondroitin sulphate chains. Therefore the size distribution of the chondroitin sulphate chains was determined by chromatography on Sephacryl S-200 of papain digests of the A1 fractions (animal A) (Fig. 3). The chromatograms, however, show that the size distribution of the chondroitin sulphate chains recovered from all the layers was very similar. Therefore other compositional parameters of the proteoglycan monomers were responsible for the variations observed.

The proteoglycan variability may be due to a changing proportion of aggregating and non-aggregating proteoglycans (Heinegård & Hascall, 1979). Therefore the A1 fractions (animal A) were chromatographed on Sepharose 2B after preincubation with hyaluronate to ensure that all proteoglycan monomers capable of interacting with hyaluronic acid would chromatograph in the void volume. The chromatograms (Fig. 4) indicate that

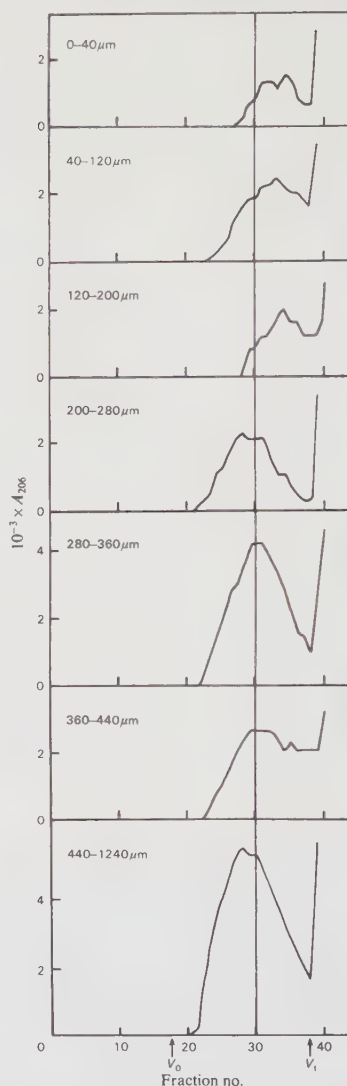


Fig. 2. Sepharose 2B chromatograms of proteoglycan monomers (reduced and alkylated A1 fractions from animal B)

The column (0.5 cm $\times$ 140 cm) was eluted with 0.25 M- Na_2SO_4 in 740 μl fractions. The A_{206} of the effluent was monitored with a LKB Uvicord S instrument. The numbers indicate depth of cartilage layers from the surface. The total-volume peak contains guanidinium chloride and dithiothreitol from reduction.

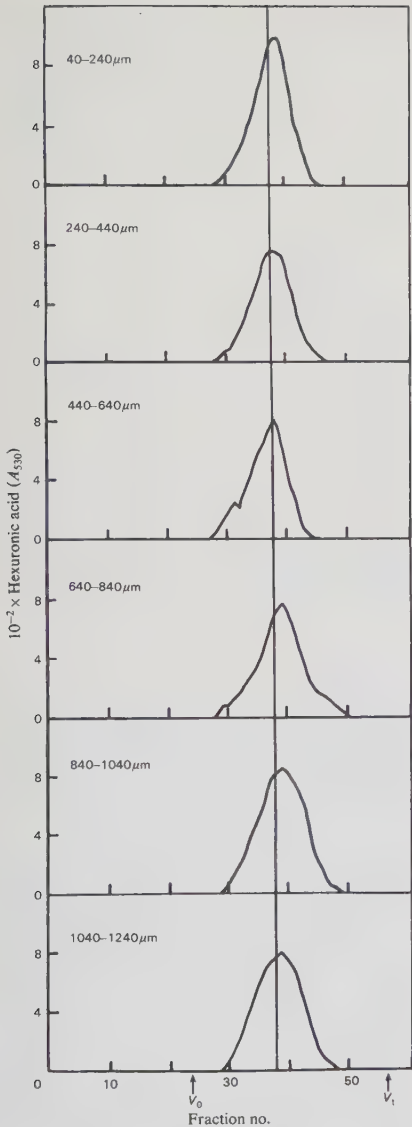


Fig. 3. *Sephacryl S-200* chromatograms of papain-digested A1 fractions from the layers indicated from animal A

The column (0.3 cm × 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μl fractions. The numbers indicate depth of cartilage layers from the surface.

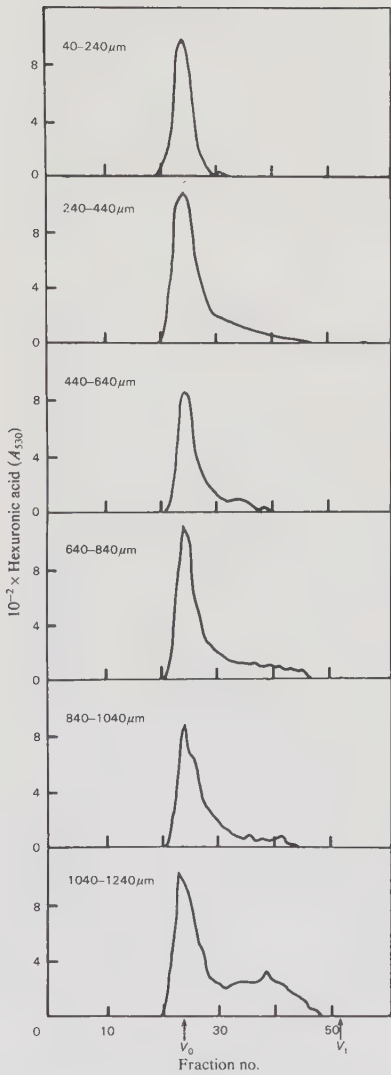


Fig. 4. *Sepharose CL 2B* chromatograms of A1 fractions from the different layers from animal A

Before chromatography the samples were incubated with 1% (w/w) of high-molecular-weight hyaluronic acid (Healon). The column (0.3 cm × 140 cm) was eluted with 0.5 M-sodium acetate, pH 7.0, in 300 μl fractions. The numbers indicate depth of cartilage layers from the surface.

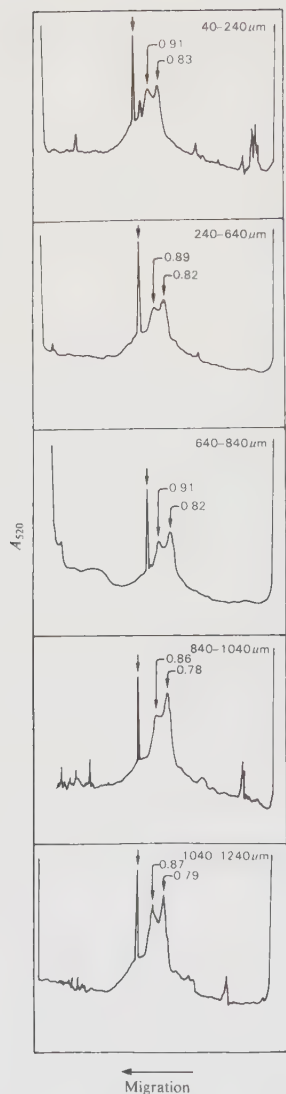


Fig. 5. Agarose/polyacrylamide-gel electrophoresis of reduced and alkylated A1 fractions from different layers.

The spectrophotometric scans at 520 nm are shown. The front marker (Bromophenol Blue) is indicated by an un-numbered arrow in each scan. The migration rate relative to that of Bromophenol Blue for the two or three components is indicated above the respective peaks. The numbers in μm indicate depth of cartilage layers from the surface.

the relative contents of aggregating proteoglycans did not change from the layers at 240–1040 μm . The top layer analysed (40–240 μm) contained only molecules capable of interacting with hyaluronic acid. The sample prepared from the proliferating zone near the cartilage–bone junction, on the other hand, contained a somewhat higher proportion of non-aggregating proteoglycans, possibly owing to changes in the cartilage matrix during cell proliferation and subsequent calcification.

Agarose/polyacrylamide-gel electrophoresis

On agarose/polyacrylamide-gel electrophoresis, cartilage proteoglycans migrate as two main components (Roughley & Mason, 1976). The scans of the agarose/polyacrylamide gel of the proteoglycans in the most superficial layer (40–240 μm) (Fig. 5) showed the two components. The scans of the preparations from the other sections showed only the two major bands. The proportion of these two bands changed so that the slower-migrating proteoglycan were somewhat more enriched with increasing depth of the cartilage (Fig. 5). The relative migration rate also decreased for both components in the deeper layers (840–1240 μm). These data indicate that there is more than one type of proteoglycan present in the articular cartilage. The smaller is enriched in the superficial layers.

General discussion

The data presented indicate that the proteoglycans isolated from the layers 0–200 μm differed from proteoglycans of deeper layers. They had a different composition, were of smaller size and all of them aggregated with hyaluronic acid. It should be pointed out, however, that there was an overlap in the chromatographic behaviour (Figs. 2 and 3). Therefore it is possible that the type of proteoglycans present in the most superficial layers (0–200 μm) may well have been present among the proteoglycans from the other layers, but then as one of several components. A small-molecular-size proteoglycan has previously been demonstrated in associative extracts of calf articular cartilage (Lipshitz *et al.*, 1976). The alterations in proteoglycan composition observed with increasing depth from 240 to 1040 μm indicate that the proteoglycan structure changes gradually from molecules containing a large chondroitin sulphate-rich region to molecules containing a smaller proportion of this region. It appears that in the intermediate sections of the cartilage the same large proportion of the molecules contained the hyaluronic acid-binding region and the keratan sulphate-rich region. The molecules found in the layer near the cartilage–bone junction had a different composition and contained a much larger proportion of non-aggregating proteo-

glycans. It is possible that some degradation of these molecules may have occurred *in vivo* and may be caused by proteinases released from the bone. The data presented indicate that the changes in proteoglycan structure with increasing age reported previously (Inerot *et al.*, 1978) are not due to losses of superficial layers of the articular cartilage. In comparison with proteoglycans isolated from nasal or tracheal cartilage of bulls of the same age, the articular-cartilage molecules are considerably smaller and have much higher content of keratan sulphate, corroborating previous data (for references see Muir, 1979). The molecules from the three tissues showed essentially the same ability to interact with hyaluronic acid and form aggregates (Hascall & Heinegård, 1974; Heinegård & Hascall, 1979). These observations are consistent with a model where the hyaluronic acid-binding region and the keratan sulphate-rich region are constant, but the chondroitin sulphate-rich region varies (Heinegård, 1977).

It should be stressed that each set of data shown in the present paper were obtained from one punch biopsy from one animal. Therefore inter-individual variability does not obscure structural differences between layers. Other sets of chromatograms obtained with samples from another animal were essentially the same, indicating that the differences observed were not unique for one animal.

Grants were obtained from the Swedish Medical Research Council (13P-5739 and 13X-05668), Alfred Österlunds stiftelse, Kocks Stiftelser, Swedish Insurance Companies Fund for Medical Research, Konung Gustav V:s 80-års fond and the Medical and Odontological Faculties, University of Lund.

References

- Axelsson, I. & Heinegård, D. (1975) *Biochem. J.* **145**, 491–500
- Harris, E. D., Jr., Parker, H. G., Radin, E. L. & Krane, S. M. (1972) *Arthritis Rheum.* **15**, 497–503
- Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4232–4241
- Hascall, V. C. & Heinegård, D. (1979) in *Glycoconjugate Research* (Gregory, J. & Jeanloz, R., eds.), vol. 1, pp. 341–374, Academic Press, New York
- Heinegård, D. (1973) *Chem. Scr.* **4**, 199–201
- Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980–1989
- Heinegård, D. & Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971–1979
- Heinegård, D. K. & Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 927–934
- Inerot, S., Heinegård, D., Audell, L. & Olsson, S. E. (1978) *Biochem. J.* **169**, 143–156
- Kempson, G. E. (1975) in *Dynamics of Connective Tissue Macromolecules* (Burleigh, P. M. C. & Poole, A. R., eds.), pp. 277–305, North-Holland Publishing Co., Amsterdam and Oxford
- Kempson, G. E., Spivey, C. J., Swanson, S. A. V. & Freeman, M. A. R. (1971) *J. Biomech.* **4**, 597–609
- Lipshitz, H., Etheredge, R. & Glimcher, M. (1975) *J. Bone Jt. Surg. Am. Vol.* **57**, 527–534
- Lipshitz, H., Etheredge, R. & Glimcher, M. (1976) *J. Bone Jt. Surg. Am. Vol.* **58**, 1149–1153
- Maroudas, A. (1979) in *Adult Articular Cartilage* (Freeman, M. A. R., ed.), 2nd edn., pp. 215–226, Pitman Medical Publishing Co., Tunbridge Wells
- Maroudas, A., Muir, H. & Wingham, J. (1969) *Biochim. Biophys. Acta*, **177**, 492–500
- Maroudas, A., Evans, H. & Almeida, L. (1973) *Ann. Rheum. Dis.* **32**, 1–9
- McDevitt, C. A. & Muir, H. (1971) *Anal. Biochem.* **44**, 612–622
- Muir, H. (1979) in *Adult Articular Cartilage* (Freeman, M. A. R., ed.), 2nd edn., pp. 145–214, Pitman Medical Publishing Co., Tunbridge Wells
- Oegema, T. R., Hascall, V. C. & Dziewiatkowski, D. D. (1975) *J. Biol. Chem.* **250**, 6151–6159
- Roughley, P. J. & Mason, R. J. (1976) *Biochem. J.* **157**, 357–367
- Scott, J. E. (1975) *Philos. Trans. R. Soc. London Ser. B*, **271**, 235–242
- Stegemann, H. & Stalder, K. (1967) *Clin. Chim. Acta* **18**, 267–273
- Stockwell, R. A. & Scott, J. E. (1967) *Nature (London)* **215**, 1376–1378

EXTRACTION AND PURIFICATION OF PROTEOGLYCANS FROM
MATURE BOVINE BONE

Ahnders Franzén^{1,2} and Dick Heinegård¹

From the Departments of Physiological Chemistry 2 and Stomatognathic
Physiology, University of Lund, Sweden

¹Department of Physiological Chemistry 2, University of Lund,
P.O.Box 750, S-220 07 Lund, Sweden

²Department of Stomatognathic Physiology, School of Dentistry, Carl
Gustavs väg 34, S-214 21 Malmö, Sweden

ABSTRACT

Proteoglycans were extracted in good yields from the mineralized matrix of ground bovine bone, using a two-step extraction procedure. Proteoglycans (6% of total), not associated with the bone mineral, were extracted at -20°C with 4 M guanidinium chloride containing proteinase inhibitors. Proteoglycans associated with the mineral, accounted for 60% of the total, were then solubilized when EDTA was added to the extraction solvent. They were fractionated and purified in presence of 4 M guanidinium chloride by CsCl density gradient centrifugations followed by chromatography on Sepharose CL4B. Further purification was obtained by chromatography on DEAE cellulose and hydroxyapatite in presence of 7 M urea. Three populations of proteoglycans and additional glycosaminoglycan peptides were obtained. The molecular dimensions of both intact molecules and of their side chains as well as their amino acid composition were different, indicating that they represent separate molecular entities.

The main proteoglycan self-aggregated in absence of 4M guanidinium chloride or 7M urea, a property which was abolished when the proteoglycan core protein was fragmented.

INTRODUCTION

Bone resist various mechanical forces, because of an ideal structural combination of its inorganic and organic constituents. In the development and growth of long bones, osteoblast lay down unmineralized bone tissue (osteoid) on remnants of calcified cartilage, a process known as endochondral ossification (for review see Bloom and Fawcett, 1975). The osteoid is mineralized after some ten days i.e. about 70 per cent of its organic matrix is replaced by calcium phosphate, mainly hydroxyapatite crystals. The mechanical properties of bone are largely determined by the size, shape and orientation of the hydroxyapatite crystals, which in turn depend on the molecular architecture of the collagen fibrils (Glimcher, 1981). More than 90 per cent of the remaining organic matrix is type I collagen (Skinner, 1979), but the matrix also contains a number of highly polyanionic molecules like phosphoproteins (Shuttleworth and Veis, 1972; Spector and Glimcher, 1972), sialoprotein (Andrews et al., 1967) and proteoglycans (Herring, 1968; Engfeldt and Hjerpe, 1976; Reddi et al. 1978). These molecules may also have effects upon the formation of the hydroxyapatite crystals.

Proteoglycans have been identified in extracts of bone (Herring, 1968; Engfeldt and Hjerpe, 1976; Reddi et al., 1978). Little is, however, known about their chemical and structural composition, as techniques allowing isolation of intact, bone specific proteoglycans in good yield have not been available. A major problem has been to liberate the proteoglycans from their association with the bone mineral. Furthermore, bone being vascularized, contains significant amounts of blood vessel wall proteoglycans. Extracts would therefore contain significant proportions of such proteoglycans. Other problems involves isolation of intact molecules, a consequence of the presence in bone of a large number of blood cells rich in proteases, capable of degrading the proteoglycans during preparation.

The aim of the present investigation has been to design procedures for extraction and purification of intact proteoglycans from the mineralized matrix of mature bovine bone. A preliminary report of some of the data has already been presented (Franzén and Heinegård, 1983).

Chemicals

Guanidine-HCl (practical grade), papain (type III, 2 x crystallized) and chondroitinase ABC were obtained from Sigma Chemical Co, St. Louis, U.S.A. Sepharose CL4B, Sepharose 6B and molecular weight calibration kits were products of Pharmacia Fine Chemicals, Uppsala, Sweden. DE52 anion exchange cellulose were bought from Whatman Chemicals Ltd, Maidenstone, Kent, U.K.. Hydroxyapatite for chromatography was from BioRad Laboratories (Richmond, California, U.S.A.).

All other chemicals used were of analytical grade. Before use the guanidine-HCl solutions were passed through a column of activated charcoal to remove UV-absorbing material. All urea solutions were passed through an anion/cation exchange resin and buffered immediately before use.

TISSUES

Diaphyses from 2 year old bulls were obtained immediately after slaughter and handled at temperatures of 0-4°C. Within an hour, muscles, bone marrow and other contaminating tissues had been removed and the bone had been crushed with a hammer, frozen in liquid N₂ and ground while still frozen using a Wiley mill. The frozen powder was fractionated at -20°C according to size using metal sieves. Particles of sizes from 60 to 120 mesh were recovered and kept at -60°C until extraction. Analytical data were, however, similar for particles of larger size, although extraction yields were somewhat lower.

ISOLATION OF PROTEOGLYCAN

Sequential extraction. In a typical experiment, 50 g (wet weight) of frozen bone powder (60-120 mesh) was extracted at -20°C for 6h with 10 volumes (500 ml) of precooled extraction medium I, i.e. 4 M guanidine-HCl 0.05 M sodium acetate buffer, pH 5.8, containing the protease inhibitors 0.1 M 6-aminoheptanoic acid, 0.005 M benzamidine chloride (Oegema et al., 1975) and 0.005 M N-ethylmaleimide, the latter mainly to prevent disulphide exchange (Heinegård et al., 1981). Extract and residue were rapidly separated by vacuum filtration through a nylon sieve and the residue was washed three times with 50 ml of ice cold extraction medium.

The residue was next extracted at 40°C for 24 hrs with 60 volumes (3000 ml) of extraction medium II, i.e. 4 M guanidinium chloride, 0.05 M Tris-HCl, pH 7.4, containing 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine chloride, 0.005 M N-ethylmaleimide and 0.25 M EDTA. The extract was clarified by centrifugation for 40 minutes at 10,000 x g (r_{av} 8.6). The pellet was washed twice with 300 ml of cold extraction medium II and again centrifuged as described above. The supernatants were pooled to form extract II.

CsCl-density gradient centrifugation. The volume of extract II was reduced to about 120 ml by ultrafiltration at 40°C using a PM-10 ultrafilter (Amicon Corp., Lexington, Mass., U.S.A.). Solid CsCl was added to the retentate to give a density of 1.35 g/ml and the preparation was centrifuged in an MSE ultracentrifuge using a 8 x 25 ml (20 ml per tube) aluminium rotor at 35,000 rpm (85,000 g, r_{av} 6.44 cm) for 60 hrs at 12°C. The tubes were emptied by gravity from the bottom into five equal fractions of 4 ml, designated D1 (bottom) to D5 (top).

Dissociative gel chromatography. A sample (20 ml) of the bottom fraction (D1) from the CsCl-density gradient centrifugation was dialyzed for 24 hrs at 40°C against two changes of 250 ml of 4 M guanidinium hydrochloride, 0.050 M sodium acetate, pH 5.8 to remove the CsCl. The retentate was then concentrated to 2 ml by ultrafiltration over an Amicon PM-10 filter and chromatographed on a Sepharose CL4B column (1.5 x 140 cm) eluted at 6 ml/hour with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8.

High-density CsCl-gradient centrifugation. The pooled fraction (4B-III) from the dissociative Sepharose CL4B gel chromatography was concentrated by ultrafiltration over an Amicon PM10 filter and the density of the retentate was adjusted to 1.42 g/ml by adding solid CsCl.

The solution was centrifuged using a Beckman 70.1 Titanium angle rotor (13 ml/tube; 2 mg/ml) at 45,000 rpm (140,000x g, r_{av} 6.1 cm) for 60 hrs and 12°C. The tubes were emptied by pumping the solution from the bottom using a peristaltic pump and fractions of 0.5 ml were collected. The densities were determined as described above.

Anion exchange chromatography. The pooled fraction (DIII containing 20 mg of material), from the second CsCl-density gradient centrifugation was dialyzed for 36 hrs at 4°C against three changes of 100 volumes of 7 M urea, 0.01 M TrisHCl, 0.1 M sodium acetate, pH 6.0. The retentate was chromatographed on a DEAE (DE52) cellulose column (1.0 x 15 cm) eluted at 4°C with 10 ml/hour of 7 M urea, 0.01 M Tris, 0.1 M sodium acetate pH 6.0. Material bound to the column was eluted with a linear gradient from 0.1 M to 1.20 M sodium acetate in the urea solution, pH 6.0.

Hydroxy apatite chromatography. A sample (10 ml, 1.5 mg/ml) of the pooled fraction (DE52-III) from the DE52 chromatography was dialyzed for 24 hrs at 4°C against two changes of 250 ml 7 M urea, 0.01 M TrisHCl, 0.001 M sodium phosphate, pH 7.4.

The retentate was applied to a hydroxyapatite column (2 x 20 cm) eluted at 14°C with 10 ml/h of 7 M urea, 0.01 M Tris, 0.001 M sodium phosphate buffer, pH 7.4. Material bound to the hydroxyapatite was eluted with a linear sodium phosphate gradient from 1 mM to 0.5 M in the urea solution, pH 7.4.

ELECTROPHORESIS

Sodium dodecyl sulphate (SDS)/polyacrylamide-gel electrophoresis.

Equally large samples of the CsCl-gradient fractions (D1 to D5) and of the pooled fractions from the Sepharose Cl4B chromatogram (CL4B I-V), respectively, were dialyzed against two changes of 1500 ml 0.010 sodium acetate/0.010 M Tris HCl, pH 8.0 for 48 hrs at 4°C. To depolymerize the glycosaminoglycan chains one half of each sample was incubated at 37°C for 6 h with chondroitinase ABC (0.01 units/mg proteoglycan dry weight). After addition of one volume of 4% SDS, 10% (v/v) mercaptoethanol in electrophoresis buffer, all samples were incubated for 2 h at 37°C, and electrophoresed on polyacrylamide gels (T=8%, C=2.5%) in the discontinuous buffer system described by Neville (1971). Proteins were stained with 0.25% (w/v) Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

Agarose-polyacrylamide gel electrophoresis. Measured samples of fractions were incubated for 18 hrs at 4°C with 9 volumes ethanol. Precipitates formed were recovered by centrifugation, suspended in 2.5 M sodium acetate and reprecipitated with ethanol to remove traces of guanidinium chloride

as described elsewhere (Paulsson et al., 1983). Before electrophoresis, the precipitates were dried, dissolved in 1% (w/v) SDS and incubated at 37°C for 2 hrs. An equal volume of 0.02% (w/v) Bromphenol blue, 60% (w/v) sucrose dissolved in electrophoresis buffer, pH 6.0 was added and the samples were electrophoresed on agarose-polyacrylamide slab gels originally described elsewhere (McDevitt & Muir, 1971) and modified by Heinegård (to be published). Gels were stained with 0.02% toluidine blue in 0.1 M acetic acid and destained in 3% acetic acid.

CHEMICAL METHODS

To determine extraction yields measured samples of the two extracts and the residue were extensively dialysed against distilled water and freeze-dried. The samples were suspended into 0.05 M sodium phosphate buffer, pH 7.0 containing 0.075 M disodium EDTA and 0.005 M 2-mercaptoethanol and digested for 24 h at 60°C with 20 μ l of a suspension of crystalline papain (25 mg protein/ml) (Hjertquist and Vejlens, 1968). Glycosaminoglycans were isolated from the digests using ion exchange chromatography on DEAE-cellulose (Whatman DE 52) as described elsewhere (Axelsson and Heinegård, 1975). Columns were eluted with water, 0.02 M HCl and 2 M HCl. The latter fraction contains the glycosaminoglycans, which were hydrolyzed in 4 M hydrochloric acid (Aristar) for 10 hrs at 100°C in tubes sealed under argon. Contents of glucosamine and galactosamine were determined using an automatic amino acid analyzer. The extraction yield was calculated from the amount of glycosaminoglycan bound hexosamine in the two extracts (I,II) and the residue.

To determine the distribution of glycosaminoglycans and proteoglycans in the CsCl density gradient fractionation of extract II, equal samples of fractions from the gradient (D1 through D5) were dialyzed against sodium chloride and then extensively against distilled water and freeze dried. One half of each fraction was digested with papain and the glycosaminoglycans were quantified as described above.

The other half of the freeze dried material was dissolved in 0.1 M sodium hydroxide and the protein content in the fractions was determined using the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Uronic acid contents of samples dissolved in 4 M guanidine or 4 M guanidine/CsCl was determined by the carbazole method of Bitter and Muir (1962).

Uronic acid contents of fractions containing 7 M urea was determined using an automated version of the carbazole method (Heinegård, 1973). Sialic acid was determined using an automated version (Lohmander et al., 1980) of the method described by Jourdain et al. (1971). To increase colour yields all urea solutions were diluted to 0.4 M urea with distilled water before quantification of sialic acid.

COLUMN CHROMATOGRAPHY

An analytical Sepharose 6B column (0.3x140 mm) was eluted with 0.5 M sodium acetate, pH 7.0 also containing bovine serum albumin (20 µg/ml) as a carrier. The column was eluted at 600 µl/h and fractions of 230 µl were collected. Samples containing 100 µg of proteoglycan were dissolved in 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 5.8 (4 mg/ml) and then dialyzed into water. One half of each sample was then freeze-dried and the other half was mixed with double concentrated elution buffer and chromatographed on the analytical Sepharose 6B column. The freeze dried samples were solubilized in 0.05 M sodium hydroxide-1 M sodium borohydride (Carlson, 1968) and incubated for 48 h at 45°C to liberate the chondroitin sulphate chains. Before gel chromatography on the Sepharose 6B column, the samples were neutralized with 1 M acetic acid.

RESULTS AND DISCUSSION

Extraction

Bone is composed of a mineralized matrix with additional components of soft connective tissue, blood vessels and bone marrow. A preparation of proteoglycans from bone, would therefore contain molecules from all these tissue components. Most connective tissue proteoglycans, however, can be extracted in good yield with 4 M guanidine chloride (Antonopoulos et al., 1974), a solvent that will not dissolve the hydroxyapatite crystals. The strategy chosen in the present paper was therefore to first extract "nonspecific" proteoglycans from pulverized bone with 4 M guanidinium

chloride and in a second step, by adding EDTA to the extracting solvent, dissolve the mineral containing bound proteoglycans. A similar approach was used by Fischer et al. (1983), who extracted subperiosteal bone pieces from immature bovine animals. To prevent proteolytic fragmentation of the macromolecules a number of protease inhibitors were included in the extraction media and importantly the extraction was performed at very low temperature, -20°C. This first extract (I) would contain any proteases present, in addition to proteoglycans not associated with the calcified matrix.

The time course of the first extraction was studied. Blood vessel wall proteoglycans were used as markers for "non-specific" proteoglycans, since these could be quantitated by radial immunodiffusion according to Mancini et al. (1965) using antibodies directed against bovine aorta proteoglycans (unpublished). Only a 3-6 hrs extraction was required to extract the nonspecific proteoglycans (Fig. 1). At that time 7% of all the proteoglycans in the bone were extracted, Table I. Subsequent extraction (II), with 4 M guanidine-HCl containing EDTA, solubilized another 60% of the proteoglycans, while 33% still remained in the insoluble residue. Similar extraction yields have previously been obtained by direct extraction with 4 M guanidine-HCl containing EDTA (Engfeldt & Hjerpe, 1976). Corroborating previous data (Hjertquist and Vejlens, 1968), the major type of glycosaminoglycans in extracts and residue were galactosaminoglycans, i.e. chondroitin sulphate and/or dermatan sulphate. The first extract as well, as the residue contained somewhat higher proportions of glucosaminoglycans than the second extract, perhaps reflecting higher contents of heparan sulphate and hyaluronic acid.

The second extract was concentrated by ultrafiltration. Some organic material (about 30% of the total) was lost during this procedure. The glucosaminoglycan to galactosaminoglycan ratio, however, remained constant, indicating no selective loss of proteoglycans.

Samples of extract II were electrophoresed on an agarose-polyacrylamide gel after ethanol precipitation. Staining with toluidine blue showed two metachromatic bands (Fig. 2). The one with highest mobility had the same electrophoretic mobility as a reference of chondroitin-6-sulphate chains from nucleus pulposus. This component did not stain with Kenacid blue,

indicating very low content of protein. It appears then, that this fast moving component represents glycosaminoglycan chains solubilized from the calcified bone matrix. The component with lower mobility showed both metachromasia and was stained with Kenacid blue for protein. It had the same electrophoretic mobility as the small proteoglycans found in many other connective tissues and could represent a proteoglycan of similar type.

The presence of glycosaminoglycan chains in extracts of connective tissues has not been described previously. Extreme care was taken to prevent proteolytic degradation during extraction, so the chains are most likely produced by a normal catabolic process. Whether the chains are derived from the type of proteoglycan present in the extract or from other types of proteoglycans present during other stages of bone formation, is not known. Noteworthy, however, when rat bone was extracted using the same procedure only the proteoglycan component was found, with no identifiable faster moving glycosaminoglycan chains (unpublished).

CsCl-density gradient centrifugation

Proteoglycans were purified by CsCl-density gradient centrifugation under dissociative conditions in 4 M guanidine-HCl, to minimize the effects of any proteases present. The major portion of the proteoglycans and glycosaminoglycans was recovered at the bottom of the gradient, while most of the protein was found at the top, Fig.3. The bottom D1-fraction ($\rho=1.42$ g/ml) contained about 55% of the galactosaminoglycans present in the gradient. The relative proportion of glucosaminoglycans increased with decreasing buoyant density, indicating a changing proteoglycan composition, Fig. 3.

Equal samples of the fractions were analyzed by SDS-polyacrylamide gel electrophoresis with and without previous removal of galactosaminoglycan side chains by digestion with chondroitinase ABC and thereby reveal the molecular dimensions of the core protein preparation (Heinegård et al., 1981). The bottom (D1) fraction contained a small amount of protein migrating with the front, while all other fractions contained several other protein components, Fig. 4. Following chondroitinase digestion of the sample, however, fraction D1 contained a new component representing the proteoglycan protein core with an apparent molecular weight of 46,000

dalton. Interestingly, proteoglycans which upon chondroitinase digestion yield core proteins with similar apparent molecular weight on SDS-PAGE have been isolated from bovine nasal cartilage (Heinegård et al., 1981) tendon (Vogel and Heinegård, unpublished) and sclera (Cöster and Fransson, 1981). Fraction D2 contained a component with the same electrophoretic mobility, which although present prior to digestion became more pronounced after chondroitinase digestion, Fig. 4.

The D1-fraction was also characterized by agarose-polyacrylamide gel electrophoresis. Two bands showed metachromasia with toluidine blue staining but only the slow moving component also stained for protein (Fig. 4) and therefore probably represents the proteoglycan.

Sepharose CL4B chromatography

The bottom D1-fraction contained the major portion of the proteoglycans as shown by glycosaminoglycan contents and by agarose-polyacrylamide gel electrophoresis. Furthermore, this fraction had a very low content of contaminating proteins and was therefore used as starting material for the purification of the proteoglycans by gel chromatography on Sepharose CL4B eluted with 4 M guanidinium chloride. The elution pattern showed a void volume peak, mainly containing nucleic acid as indicated by its high absorbance at 260 nm (Fig. 5). The proteoglycans (about 90% of the glycosaminoglycans in the chromatogram) eluted as a broad retarded peak, which contained more than one protein component. The colour yield in the carbazole reaction obtained with the void volume component is probably nonspecific, since it corresponded to only some 2% of the total glycosaminoglycan-hexosamine.

Agarose polyacrylamide electrophoresis of equal samples of the pooled fractions (Fig. 5 insert) showed that the void volume peak contained three weakly staining components, with lower electrophoretic mobility than fraction III, possibly representing a small proportion of larger proteoglycans. The major fraction of proteoglycans (III) contained two components with mobilities corresponding to small proteoglycans in cartilage (Heinegård et al., 1981) and to chondroitin sulphate chains, respectively.

The fractions (I-V) were also electrophoresed on SDS polyacrylamide gels with and without prior chondroitinase ABC digestion as described above (data not shown). No proteins were observed in the gels corresponding to fractions I and II. Fraction III contained a major component which after digestion with chondroitinase had an apparent molecular weight of 46,000 dalton. This component did not enter the gel if not digested with chondroitinase, and therefore represents the proteoglycan protein core. Fraction V contained protein migrating with the electrophoresis front. Since this protein was recovered in the high density bottom fraction after CsCl-gradient centrifugation it is polyanionic, possibly representing phosphoprotein.

High density CsCl-gradient centrifugation

A second CsCl-density gradient centrifugation was used to separate the proteoglycans from the chondroitin sulphate chains. The partially purified proteoglycans (fraction III, Fig. 5) were further centrifuged in CsCl containing 4 M guanidium chloride using a somewhat higher starting density (1.42 g/ml) and higher centrifugation rate when compared with the initial centrifugation. The bottom, high density fractions contained a component rich in uronic acid and poor in protein, (Fig. 6). It accounted for about 20% of the total hexuronic acid in the gradient and contained glycosaminoglycan chains and little or no proteoglycan as shown by agarose-polyacrylamide (Fig. 6 insert) and SDS-polyacrylamide gel electrophoresis after chondroitinase digestion (data not shown). The remainder of the uronic acid distributed in a broad peak extending over the bottom two thirds of the gradient (Fig. 6).

The gradient fractions were also analyzed for contents of sialic acid. A component very rich in sialic acid and partially separated from the main hexuronic acid containing peak was found in fractions of lower densities.

Anion exchange chromatography

An attempt was made to separate the sialic acid containing component from the proteoglycan in the pooled fraction III of the CsCl-gradient by using ion exchange chromatography.

Fraction III was dialyzed into 7 M urea, 0.1 M sodium acetate, pH 6.0 and chromatographed on DEAE-cellulose (DE52). Bound material was eluted with a linear gradient of sodium acetate from 0.1 M to 1.2 M.

Two well separated peaks were observed, the leading eluted at 0.45 M sodium acetate and apparently containing the sialic acid rich component but no proteoglycan, since it contained no uronic acid and no proteoglycan was observed on agarose polyacrylamide gel electrophoresis (Fig. 7 insert). This sialoprotein was not further characterized. The second peak, eluted at 0.7 M sodium acetate contained the proteoglycan (Fig. 7). Surprisingly agarose-polyacrylamide gel electrophoresis showed that it also contained some chondroitin sulphate chains (Fig. 7 insert), although no such chains could be identified in the material used for chromatography (Fig. 6 insert). It is possible that some interactions can only be dissociated in urea solutions.

Hydroxyapatite chromatography

The proteoglycans (DE52-III) were further fractionated into subclasses by chromatography on hydroxyapatite eluted in denaturing conditions of urea with a linear sodium phosphate gradient. Some protein rich material was not bound to the hydroxyapatite matrix and eluted from the column before the gradient was started (Fig. 8). The bound material chromatographed in two main fractions. One component was eluted in a sharp peak at about 0.1 M sodium phosphate. It accounted for about 50% of the hexuronic and about 25% of the protein (absorbance at 280 nm) in the chromatogram (Fig. 8). The other fraction required higher salt concentration for elution and was more heterogeneous. It appeared to contain at least two components. Surprisingly they have higher protein to hexuronic acid ratios than that observed for the leading component. All components bound to hydroxyapatite, i.e. fraction II, III and IV, contained proteoglycans as shown by agarose-polyacrylamide gel electrophoresis (Fig. 8 insert). Three fractions, HTP II, III and IV were pooled as indicated in the figure. From a typical chromatogram 8.0 mg, 2.4 mg and 2.7 mg, respectively, were recovered.

It should be stressed that chromatography on hydroxyapatite proved to be a prerequisite for removing small amounts of sialoprotein present in the proteoglycan fraction recovered from the DEAE-cellulose chromatography

(data on the specific quantification of the sialoprotein using ELISA is not included in this presentation). Interestingly the sialoprotein showed higher affinity for the hydroxyapatite than did the proteoglycan.

Composition of the proteoglycan fractions

The analytical data indicate that the calcified matrix of bone contains at least three types of proteoglycans, i.e. fractions II, III and IV from the chromatography on hydroxyapatite, and additional chondroitin sulphate peptides, i.e. the high density fraction (CsCl I) from the second CsCl gradient. These four fractions were further analyzed, Table II. The major proteoglycan fraction (HTP II) had a protein content of 39%. The predominating amino acids were leucine, aspartic acid/asparagine and glutamic acid/glutamine, indicating structural similarities with the small proteoglycans from other sources. Its content of hexosamine is 14%, of hexuronic acid 16,2% and of sialic acid 0,8%. The major proportion of the hexosamines was galactosamine, indicating a high content of chondroitin sulfate.

The other two proteoglycan fractions had lower protein contents and also lower hexosamine contents compared with the major proteoglycan. Interestingly their contents of sialic acid were higher (Table II). These two preparations had higher glutamic acid/glutamine and proline contents, while they contained less leucine compared with the major proteoglycan. Indeed their amino acid composition was similar to that of the chondroitin sulphate peptides, although the latter expectedly had a very high ratio of hexosamine to protein (Table II). The high relative protein contents of fractions HTP III and IV, however, makes it unlikely that these components represents degradation products of the major proteoglycan fraction.

Size distributions of the proteoglycan fractions

Chromatography on Sepharose 6B in 0.5M sodium acetate, pH 7.0, showed that the major component, i.e. HTP II, eluted in two peaks (Fig. 9b) one eluting in the void volume and the other well included. Since this material chromatographs in well included symmetrical peak under dissociating conditions of 4 M guanidine-hydrochloride, it appears that the proteoglycans can form self-aggregates. Further proof was obtained by rechromatographing material from the two peaks, again under associative conditions. Both again gave rise to one void volume peak and one included peak (data

The other two proteoglycan fractions chromatographed in major included peaks (Fig. 9c,d) corroborating their different nature. Fraction HTP III, however, gave a minor void volume component, possibly representing some contamination with the major proteoglycan. Furthermore, alkaline borohydride treatment to liberate the chondroitin sulphate side chains and subsequent gelchromatography gave further proof of the different nature of the proteoglycans. The major proteoglycan fraction, HTP II, contained side chains considerably larger ($K_{av}=0.52$) than those of the other proteoglycan fractions ($K_{av}=0.67$), which in turn had side chains similar in size to those of major cartilage proteoglycans. The chondroitin sulphate chains in the chondroitin sulphate peptide fraction (CsCl I) had a size similar to that of the major proteoglycans (Fig. 9a), perhaps indicating that they are derived from these molecules by their normal turnover.

ACKNOWLEDGEMENTS

Grants were obtained from the Swedish medical research council (05668), Folksam's yrkesskadors stiftelse, Kock's stiftelser, Österlunds stiftelse, Konung Gustaf V:s 80-års fond and the Faculties of Medicine and Odontology, University of Lund.

REFERENCES

- Andrews, A.T. deB, Herring, G.M. and Kent, P.W. (1967) *Biochem. J.* 104, 705-715
- Antonopoulos, C.A., Axelsson, I., Heinegård, D. and Gardell, S. (1974) *Biochim. Biophys. Acta* 338, 108-119
- Axelsson, I. and Heinegård, D. (1975) *Biochem. J.* 145, 491-500
- Bitter, T. and Muir, H. (1962) *Anal. Biochem.* 4, 330-334
- Bloom, W. and Fawcett, D.W. A textbook of Histology. 10th ed. pp. 262-279. Philadelphia. Saunders, 1975.
- Carlson, D.M. (1968) *J. Biol. Chem.* 243, 616-626
- Cöster, L. and Fransson, L.-Å. (1981) *Biochem. J.* 193, 143-153
- Engfeldt, B. and Hjerpe, A. (1976) *Acta Path. Microbiol. Scand.*, sect. A 84, 95-106
- Fischer, L.W., Termine, I.D., Dejter, S.W., Whitson, S.W., Yanagishita, M., Kimura, J.H., Hascall, V.C., Kleinman, H.K., Hassel, J.R. and Nilsson, B. (1983) *J. Biol. Chem.* 258, 6588-6594
- Franzén, A. and Heinegård, D. Isolation and characterization of proteoglycans from mature bovine bone. Abstract at the 7th International Symposium on Glycoconjugates, Lund-Ronneby, July 17-23, 1983.
- Glimcher, M.J. (1981) in *The Chemistry and Biology of mineralized connective tissues*. pp. 617-673, Veis, A. ed. North-Holland, Amsterdam.
- Heinegård, D. (1973) *Chem. Scr.* 4, 199 201
- Heinegård, D., Paulsson, M., Inerot, S. and Carlström, C. (1981) *Biochem. J.* 197, 355-366
- Herring, G.M. (1968) *Biochem. J.* 107, 41-49
- Hjertquist, S.-O. and Vejlens, L. (1968) *Calcif. Tiss. Res.* 2, 314-333
- Jourdain, G.W., Dean, L. and Roseman, S. (1971) *J. Biol. Chem.* 246, 430-435
- Lohmander, S., DeLuca, S., Nilsson, B., Hascall, V.C., Caputo, C.B., Kimura, J.H. and Heinegård, D. (1980) *J. Biol. Chem.* 255, 6084-6091
- Lowry, O.H., Rosebrough, N., Farr, L. and Randall, R. (1951) *J. Biol. Chem.* 193, 265-275
- Mancini, G., Carbonava, A.O. and Heremans, J.F. (1965) *Immunochemistry* 2, 235 254
- McDevitt, C. and Muir, H. (1971) *Anal. Biochem.* 44, 612-622
- Neville, D. (1971) *J. Biol. Chem.* 246, 6328-6334
- Oegema, T.R., Hascall, V.C. & Dziewiatkowski, D.D. (1975) *J. Biol. Chem.*

- Paulsson, M., Sommarin, Y. and Heinegård, D. (1983) *Biochem. J.* 212, 659-667
- Reddi, A.H., Hascall, V.C. and Hascall, G.K. (1978) *J. Biol. Chem.* 253, 2429-2436
- Shuttleworth, A. and Veis, A. (1972) *Biochim. Biophys. Acta* 257, 414-420
- Skinner, H.C.W. (1979) *The Scientific Basis of Orthopaedics*, pp. 105-133.
- Albright J.A. and Brand, R.A. eds. *Appelton-Century-Crofts*, New York
- Spector, A.R. and Glimcher, I.M. (1972) *Biochim. Biophys. Acta* 263, 593-603

Table I. Distribution of proteoglycans in extracts and residue. Ground bone tissue (50 g, wet weight) was extracted with the two step extraction procedure. (For experimental details see Material and methods.) Mean of three separate experiments. Proteoglycans are expressed as glycosaminoglycan-bound hexosamine (Means S.E.M.).

Fraction	glucosaminoglycans	Extraction yield (% hexosamines in extracts and residue)
	galactosaminoglycans (w/w)	
Extract I (GuHCl)	0.093	7.7 $\pm$ 2.0
Extract II (GuHCl-EDTA)	0.024	61.0 $\pm$ 0.7
Residue	0.042	31.3 $\pm$ 2.8

Table II. Amino acid and carbohydrate composition of the three bone proteoglycan populations (fraction HTP II, HTP III, HTP IV, Fig. 7) and the additional glycosaminoglycan peptide (fraction CsClI, Fig. 5). Expressed as res./1000 res.

	CsClI	HTPII	HTPIII	HTPIV
Hypro		Not detected		
Asp	125	128	126	145
Thr	53	45	52	66
Ser	100	76	102	106
Glu	160	105	158	205
Pro	79	77	72	54
Gly	114	86	114	126
Ala	48	49	53	45
Cys		Not determined		
Val	33	52	41	26
Met		Not determined		
Ile	56	58	36	27
Leu	81	128	83	46
Tyr	19	29	21	24
Phe	21	32	25	16
His	28	31	26	24
Lys	41	69	50	49
Arg	44	36	44	41
Protein*	7.9	39.9	25.5	28.8
(% of dry weight)				
Hexuronic acid	31.1	16.2	10.0	8.9
(% of dry weight)				
Sialic acid	0.4	0.8	1.2	2.4
(% of dry weight)				
Hexosamines	19.9	13.9	6.2	4.4
(% of dry weight)				

*From amino acid analysis.

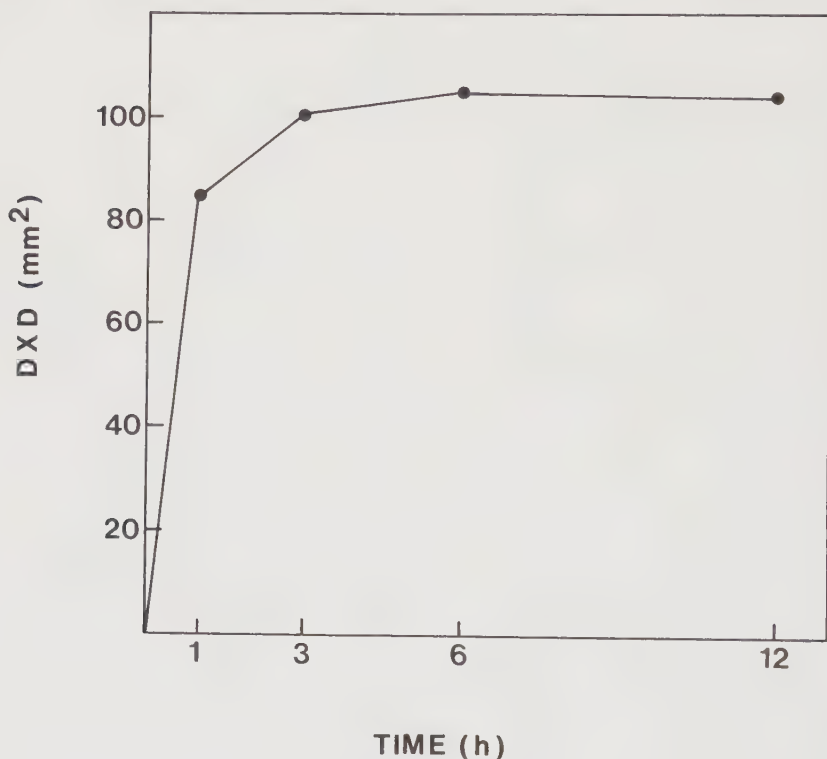


Fig. 1. Extraction of "non-specific" proteoglycans.

A diagram showing the time course of extraction from mature bovine bone of proteoglycans immunoreactive with antibodies to aorta proteoglycans. Ground bovine bone was extracted for various times at -20°C with 4 M guanidine HCl including protease inhibitors. The amount of extracted immuno-reactive proteoglycans was determined by a radial immuno diffusion according to Mancini et al. (1965), using identical aliquots of the various bone extracts.

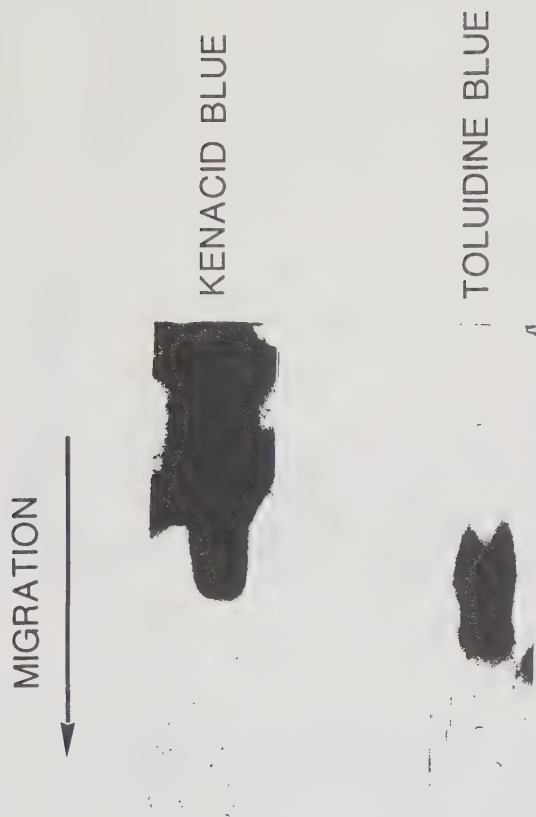


Fig. 2. Analysis of extract containing "specific" proteoglycans.

Agarose-polyacrylamide gel electrophoresis of measured samples of the second extract (4 M guanadinium chloride-EDTA) of bovine bone tissue. The gels was stained with Kenacid Blue R (left) for visualisation of proteins and with Toluidine Blue (right) for visualisation of glycosaminoglycans. For further experimental details see Materials and Methods.

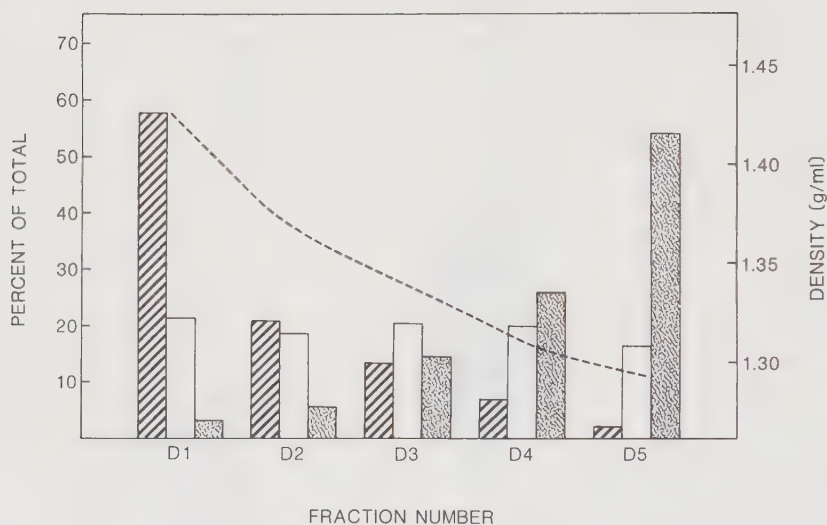


Fig. 3. CsCl-density gradient centrifugation.

Cesium chloride density gradient centrifugation of the second extract (4 M Guanidine-EDTA) of the ground bovine bone tissue. The centrifugation tubes were emptied from the bottom into five equal fractions, D1 (bottom) to D5 (top). Measured samples of the gradient fractions were analyzed for content of proteins (stippled), glucosaminoglycans (white) and galactosaminoglycans (hatched). For experimental details see Materials and Methods.

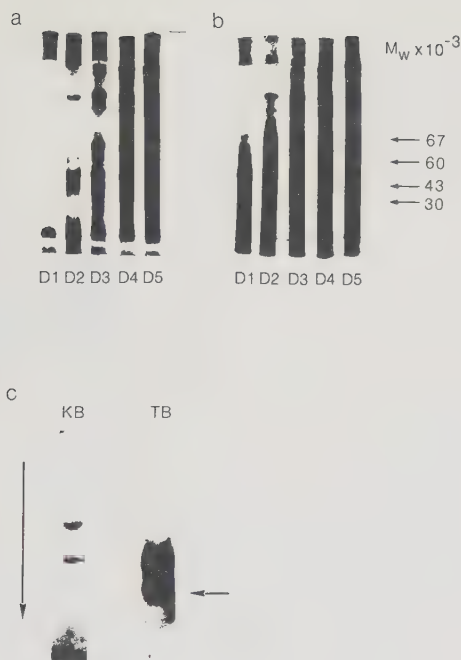


Fig. 4. Sodium dodecyl sulphate polyacrylamide and agarose-polyacrylamide gel electrophoresis of samples from the CsCl gradient centrifugation of bone extract.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis of identical samples from the CsCl density gradient fractions of the second bone extract. Before electrophoresis the gradient fractions were incubated at 37°C for 6 h without (a) and with (b) previous addition of chondroitinase ABC. Prior to electrophoresis the samples were reduced with 2-mercaptoethanol. For details see Materials and Methods.

Agarose-polyacrylamide gel electrophoresis (c) of identical samples of the bottom fraction (D1) obtained from the CsCl density gradient centrifugation. The gel was stained with Kenacid Blue R (KB) or Toluidine Blue (TB). The horizontal arrow indicates the migration distance of the chondroitin-6-sulphate chains used as reference. For experimental description see Materials and Methods.

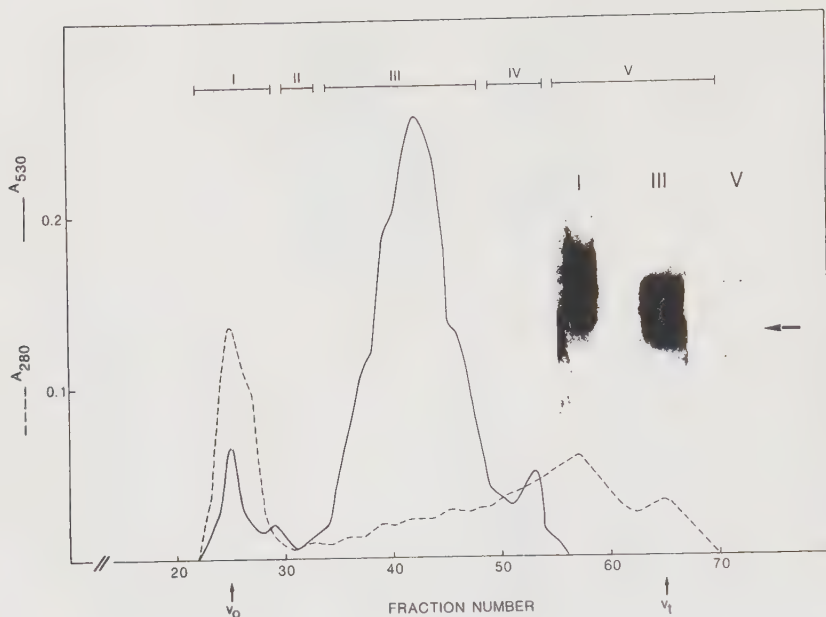


Fig. 5. Sepharose CL4B chromatography of partially purified proteoglycans.

Sephacrose CL4B chromatogram in 4 M Guanidine-HCl of the bottom fraction (D1) obtained from the CsCl density gradient centrifugation. The fractions were analyzed for contents of protein (measured as absorbance at 280 nm and hexuronic acid (carbazole reaction A_{530} nm —). The chromatogram was pooled into five fractions (1-5) as indicated in the Figure. For further experimental details see Materials and Methods.

Fig. 5 insert.

Agarose polyacrylamide gel electrophoresis of measured aliquots of the fractions (I,III,V) obtained from the Sepharose CL4B chromatogram. After electrophoresis the gel was stained with Toluidine Blue for visualization of glycosaminoglycans. The horizontal arrow indicate the migration of the standard (chondroitin-6-sulphate chains). The experiments are further described in Materials and Methods.

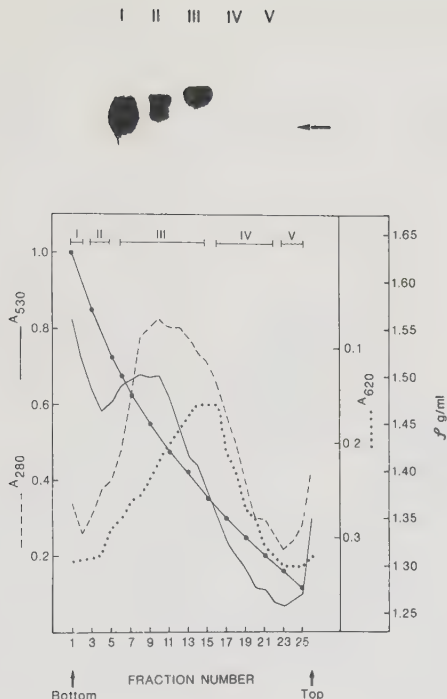


Fig. 6. Cesium chloride density gradient centrifugation in 4 M guanidine HCl of fraction (III) obtained from the Sepharose CL4B chromatography.

After centrifugation, the tubes was emptied by pumping the solution from the bottom using a peristaltic pump. The density of every second fraction was determined by pycnometry (·-·-·). The fractions were analyzed for content of protein (absorbance at 280 nm ---), hexuronic acid (absorbance at 530 nm —), and sialic acid (absorbance at 620 nm). The CsCl gradient was poled into five (I-V) fractions as indicated in the Figure. For further details see Materials and Methods.

Fig. 6 insert

At the top is shown the agarose-polyacrylamide gel electrophoresis of identical samples from the fractions (I-V) of the second CsCl density gradient centrifugation. The gel was stained with Toluidine Blue for visualizaton of glycosaminoglycans. The arrow indicate the position of the chondroitin-sulphate chains. For experimental details see Materials and Methods.

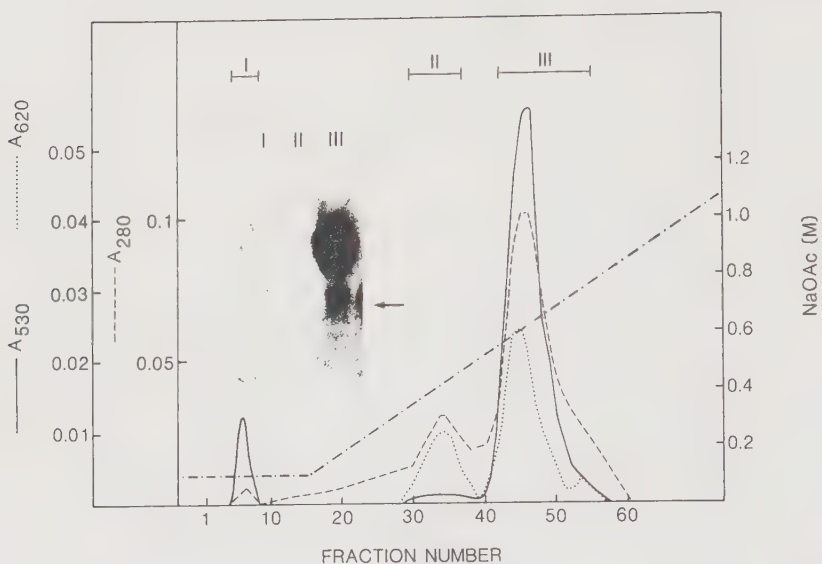


Fig. 7. DEAE ion exchange chromatography in 7 M urea of fraction (III) obtained from the second CsCl density gradient centrifugation.

The fractions were analyzed for content of proteins (absorbance at 280 nm ---), hexuronic acid (absorbance at 530 nm —) and sialic acid (absorbance at 620 nm). The slope of the sodium acetate gradient (---) was determined by conductivity measurements, using standards of known sodium acetate concentration. The chromatogram was pooled into three fractions (I-III) as indicated in the Figure. The experimental protocol is described in Materials and Methods.

Fig. 7 insert.

Agarose-polyacrylamide gel electrophoresis of measured aliquotes of the fractions (I-III) obtained from the chromatogram. The gel was stained with Toluidine Blue for visualization of glycosaminoglycans. The arrow indicates the position of chondroitin sulphate chains.

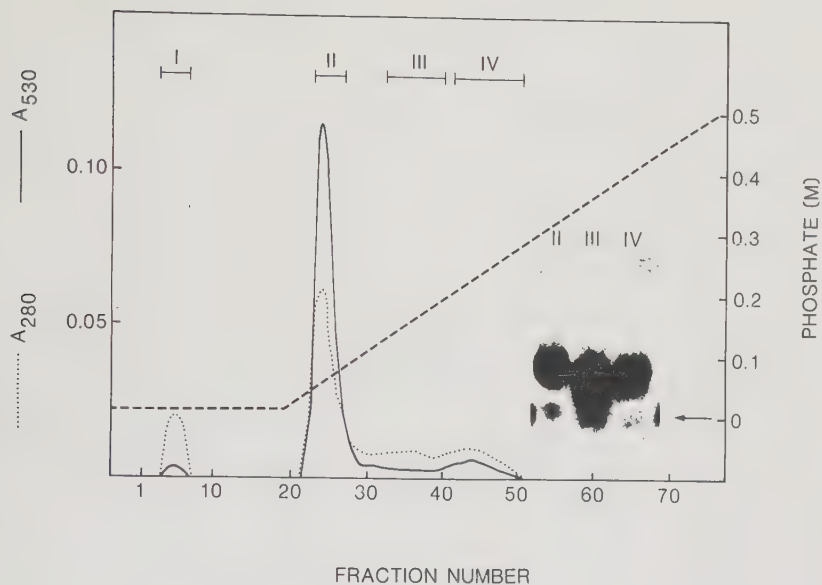


Fig. 8. Hydroxyapatite chromatography in 7 M urea of fraction (III) from the DE52 anion exchange chromatography.

The column was eluted with a linear sodium phosphate gradient. The fractions were analyzed for contents of protein (absorbance at 280 nm) and hexuronic acid (absorbance at 530 nm ———). The chromatogram was pooled into four fractions (I-IV) as indicated in the Figure. For experimental details see Materials and Methods.

Fig. 8 insert.

Agarose-polyacrylamide gel electrophoresis of identical aliquotes of the fractions (I-IV) from the hydroxyapatite chromatogram. The gel was stained with Toluidine Blue to visualize the glycosaminoglycans. The arrow indicates the position of chondroitin sulphate chains.

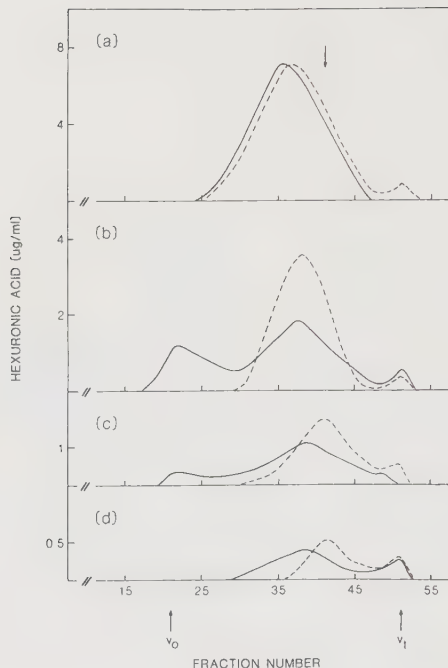


Fig. 9. Gel chromatography of proteoglycan fractions and chondroitin sulphate peptides.

Analytical Sepharose 6B chromatography in 0.5 M sodium acetate, pH 7.0 of the various glycosaminoglycan containing fractions isolated from the calcified matrix of bovine bone.

(a): CsClI (bottom fraction. Fig. 6) and the fractions obtained from the hydroxy apatite chromatogram. (b): HTPII, (c): HTPIII, (d): HTPIV.

The fractions were analyzed for content of hexuronic acid of the intact molecules (solid line) and of the alkali-borohydride treated molecules (interrupted line). The elution position of alkali-borohydride treated cartilage proteoglycan monomers is indicated with the arrow. For further experimental details see Materials and Methods.

CHARACTERIZATION OF PROTEOGLYCANS FROM THE CALCIFIED MATRIX
OF BOVINE BONE

Ahnders Franzén^{1,2} and Dick Heinegård¹

Department of Physiological Chemistry 2 and Stomatognathic Physiology,
University of Lund, Sweden

¹Department of Physiological Chemistry 2, University of Lund, P.O. Box
750, S-220 07 Lund, Sweden

²Department of Stomatognathic Physiology, School of Dentistry, Carl
Gustavs v. 34, S-214 21 Malmö, Sweden

ABSTRACT

The proteoglycans characterized were those isolated from the calcified matrix of mature bovine bone (Franzén and Heinegård, accompanying paper). The average molecular weight of the bone proteoglycan is 75000 dalton determined by sedimentation-equilibrium centrifugation in 4 M guanidinium chloride. Its sedimentation coefficient ($S_{0,20,w}^0$) is 3.04S. The apparent molecular weight of its core protein is 46000 dalton, estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the chondroitinase ABC digested proteoglycan. A more likely molecular weight of the core protein is 30000 dalton, as calculated from the molecular weight and the protein content (40%) of the proteoglycan.

The bone proteoglycan contains one or probably two chondroitin sulphate chains each with a molecular weight of 33700 dalton (M_w) and several oligosaccharides both of the N-glycosidically and O-glycosidically linked type

Antibodies against the homogenous bone proteoglycans were raised in rabbits. An ELISA was developed, which allowed specific quantitation of bone proteoglycans at nanogram levels. The specificity of the antibodies was tested using the ELISA. The bone proteoglycan showed partial cross-reactivity with the small proteoglycan of cartilage. The antibodies were used to localize immunoreactivity of bone proteoglycans by indirect immunofluorescence in frozen sections of foetal bovine epiphyseal growth plate. The fluorescence was entirely found in the primary spongiosa and no fluorescence was found among the hypertrophied chondrocytes nor in the region of provisional calcification.

INTRODUCTION

Three populations of proteoglycans having different composition and additional glycosaminoglycan peptides have been isolated from the calcified matrix of mature bovine bone (Franzen and Heinegård, accompanying paper). The composition of the proteoglycans differ. Their glycosaminoglycan chains were of different size, indicating non-identity. The core protein of the major proteoglycan fraction had an apparent molecular weight of 46000 estimated by SDS-polyacrylamide gel electrophoresis of chondroitinase ABC digests.

Proteoglycans with core proteins showing similar apparent molecular weights and with similar amino acid composition have also been identified in extracts of cartilage (Heinegård et al., 1981), cervix (Uldbjerg et al. 1982), cornea (Axelsson and Heinegård, 1975) and sclera (Cöster and Fransson, 1981). Such small proteoglycans have so far been identified in all connective tissues studied and may represent a ubiquitous proteoglycan type. It should be stressed, however, that the small proteoglycans differ, i.e. some like those from sclera and tendon contain dermatan sulphate chains with high contents of iduronic acid, while those from cartilage contain chondroitin sulphate chains with no iduronic acid. The present paper was initiated to characterize the major population of bone proteoglycans with respect to its structure, composition and to develop an immunoassay and to test some immunological relationships with the small proteoglycan of cartilage.

MATERIAL AND METHODS

Molecular weight calibration kit, alkaline phosphatase substrate (Naphthol-As-Mx-phosphate), chondroitinase AC II and ABC were products of Sigma Chemical Co., St. Louis, MO, USA. Superose CL-6B came from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio Gel P 10 was obtained from BioRad laboratories, Richmond, CA, USA and DE-52 anion exchanger was bought from Whatman Chemicals Ltd, Maidstone, England.

The proteoglycans to be characterized represent the major component (HTP II) extracted from the calcified matrix of bone and purified as described in an accompanying paper (Franzén and Heinegård).

Chemical Methods

The glycosaminoglycans and the oligosaccharides were quantitated separately. They were liberated from the proteoglycan by alkalineborohydride treatment (5 mg/ml) in 0.05 M sodium hydroxide, 1 M sodium borohydride for 48 h at 45°C (Carlson, 1968). After neutralisation by addition of 10 M acetic acid, the samples were freeze-dried, dissolved at 0.5 mg/ml in distilled water and chromatographed on DE-52 cellulose columns as described elsewhere (Inerot and Heinegård, 1983). The oligosaccharides were eluted from the columns with several volumes of distilled water followed by 0.6 M pyridine-acetate pH 6.0. The glycosaminoglycans were then eluted with 2 M HCl. Contents of hexosamines in the glycosaminoglycan and oligosaccharide fractions were determined with an automatic amino acid analyzer after hydrolysis of samples in 4 M HCl (Aristar) for 10 h at 100°C in tubes sealed under Argon. The protein content (of proteoglycan dry weight) was calculated from the sum of the amino acids (see accompanying paper). Hexuronic acid contents of samples were determined by an automated version of the carbazole method (Heinegård, 1973). Sialic acid contents were determined by an automated version (Lohmander et al. 1980) of the method described by Jourdain (1971). Hydroxyproline was quantified according to Stegeman and Stalder (1967).

Analytical ultracentrifugation

The apparent molecular weight of the bone proteoglycan was determined by sedimentation-equilibrium centrifugation in 4 M GuHCl using the meniscus-depletion method described by Yphantis (1964). Bone proteoglycan was dissolved at 5 mg/ml in 4 M guanidinium chloride (ultrapure: BRL, Bethesda, MD, USA) buffered with 5 mM Tris-HCl pH 7.0 and dialyzed against 100 volumes of the guanidinium chloride solution for 48 h at 4°C. The sample was diluted to final concentrations of 0.15 mg/ml, 0.30 mg/ml, 0.45 mg/ml and 0.60 mg/ml by adding weighed aliquots of the dialysate. Centrifugation was performed in an MSE Centriscan analytical ultracentrifuge at 20°C and 17000, 19000 and 21000 rev/min. A cushion of fluorocarbon oil (FC 70, 3M, Minneapolis, MN, USA) was included to permit optimal optical resolution also at the bottom of the cells. The distribution of the material was analyzed both with the schlieren system and by monitoring the absorbance at 280 nm. Sedimentation coefficients were determined in separate centrifugations at 55000 rpm of samples diluted to 0.55 mg/ml, 1.20 mg/ml, 1.80

mg/ml and 2.40 mg/ml. The sedimentation was followed by using the schlieren system and scans were automatically taken at regular time intervals. The sedimentation coefficients were calculated from the formulas given in the MSE manual (Technical publication, supplement 73) and the apparent S^0 -value was corrected to 20°C and water.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS-PAGE)

Prior to electrophoresis, samples were dissolved in 4 M guanidinium chloride, 0.05 M sodium acetate pH 5.8 (4 mg/ml) for 18 h at 4°C and subsequently dialyzed for 48 h at 4°C against distilled water. The retentate was adjusted to 20 mM Tris-HCl, pH 8.0, by addition of a ten times concentrated buffer. Prior to electrophoresis one half of each sample was digested with chondroitinase ABC (0.01 units/mg proteoglycan dry weight) for 6 h at 37°C. All samples were then mixed with an equal volume of the sample cocktail described by Neville (1971), containing 10% of 2-mercaptoethanol and incubated for 2 h at 37°C. Electrophoresis was performed into polyacrylamide gels (T=8%, C=2.5%) for 7 h at 15°C in a discontinuous buffer system (Neville, 1971). Proteins in the gels were stained with Kenacid Blue R (BDH Chemicals, Poole, Dorset, U.K.).

Molecular weight of the glycosaminoglycan chains

The glycosaminoglycans were liberated from the proteoglycan core protein by alkaline-borohydride treatment as described above and freeze-dried. The sample was dissolved in 0.25 M sodium sulphate, pH 7, and chromatographed on Superose CL-6B (0.6 x 140 cm), eluted with 0.25 M sodium sulphate pH 7.0. The absorbance of the column effluent at 206 nm was monitored using an Uvicord S (LKB, Sweden). Molecular weights were calculated as described by Wasteson (1971).

Characterization of the oligosaccharides and glycosaminoglycans

A sample of the purified bone proteoglycan (1.4 mg) was treated with alkaline-borohydride as described in chemical methods. The freeze-dried material was dissolved in 1 M pyridine-acetate, pH 7.0, and chromatographed on a column (0.6 x 140 cm) of Bio-Gel P 10 eluted with 1 M pyridine-acetate, pH 7.0. After chromatography, the fractions were freeze-dried, dissolved in 0.5 ml of distilled water and analyzed for contents of hexose with the orcinol method (Kesler, 1967). The elution positions of hyaluronic acid oligomers (prepared according to Hascall and Heinegård, 1974)

were used as a reference. Fractions representing each peak were pooled and freeze-dried. These pools were then dissolved in measured volumes of distilled water and weighed samples were used for determination of contents of hexosamines (see chemical methods). Other weighed samples were used for estimation of the natural sugar composition after hydrolysis of samples in 2 M trifluoroacetic acid for 3 h at 100°C in sealed tubes. A Biorad HPX-87P carbohydrate column (BioRad laboratories, Richmond, CA, USA) was eluted with water. The effluent was monitored for contents of carbohydrate using the orcinol reaction applied to a Technicon Autoanalyzer (Lohmander, personal communication).

A sample (50 µg) of the void volume peak of the BioGel P10 chromatography, containing the glycosaminoglycans, was dissolved in 50 µl of 0.1M Tris-HCl buffer, pH 8.0. Chondroitin sulphate was depolymerized by incubation with chondroitinase ACII (0.06 nominal units/mg) for 5h at 37°C. Concentrated sodium acetate was then added to a final concentration of 0.5 M and the digest was chromatographed on a column of Sephadex G50 (0.5 x 140 cm) eluted at 4°C with 0.5M sodium acetate, pH 7.0. Fractions were monitored for content of hexuronic acid with an automated version of the carbazole procedure (Heinegård, 1973).

Production of antibodies

The purified bone proteoglycan was used for raising antibodies in rabbits. Immunization was done subcutaneously in the neck with 1 mg of bone proteoglycan in Freund's Complete adjuvant (DIFCO laboratories, Michigan, USA). Monthly booster injections of 0.5 mg of the proteoglycan in Freund's Incomplete Adjuvant (DIFCO laboratories, Michigan, USA) were given until a satisfactory titer was obtained. Antibodies directed against the bone proteoglycans was detected with an ELISA technique.

ELISA (Enzyme-Linked Immuno-Sorbent Assay)

Coating of polyvinyl microtiter plates (Dynateck M29) was done overnight with 200 µl/well of a solution (1 µg/ml) of bone proteoglycans in 0.05 M sodium carbonate, pH 9.6. The proteoglycans had been digested with chondroitinase ABC (0.01 units/mg proteoglycan) prior to use. The antigen solution to be assayed was preincubated overnight in incubation buffer, i.e. 0.15 M sodium chloride, 5 mM sodium phosphate (PBS), 0.05% Tween 20, pH 7.4 to which had been added an equal volume of antibody diluted at

1:2000 with incubation buffer. After coating, the wells were extensively rinsed with PBS-0.05% Tween 20 (PBS-Tw) to remove unbound proteoglycan. Samples (200 μ l) of preincubated antigen-antibody mixture were then pipetted into the wells coated with bone proteoglycan and available antibodies were allowed to react for 1 h at 20°C. After extensive rinsing with PBS-Tw, 200 μ l of swine anti-rabbit IgG antibodies conjugated with alkaline phosphatase (Orion Diagnostica, Helsinki, Finland) diluted at 1:200 with incubation buffer and supplemented with 2 mg/ml of bovine serum albumin were added to each well and allowed to bind to the rabbit antibodies bound to the wall of the well. After 4h at 20°C, the wells were rinsed with PBS-Tw, 200 μ l of alkaline phosphatase substrate (Naphthol-AS-MX-phosphate) in 1 M diethanolamine, 0.5 mM magnesium chloride pH 9.8 was pipetted into each well. Incubation was performed at 20°C for 1 h and the absorbance at 405 nm was measured using a Titertec Multiscan filterphotometer (Flow Laboratories, USA).

The specificity of the antibodies were tested. Powdered bovine diaphysis was extracted as described (Franzén and Heinegård, accompanying paper). The extract from the calcified bone matrix (guanidinium chloride-EDTA) was extensively dialyzed at 4°C against 7 M urea, 0.01 M Tris-HCl 0.1 M sodium acetate, pH 6.0. The retentate was chromatographed on a DEAE-cellulose (DE52) ion exchange column (11 mm x 125 mm) eluted with a sodium acetate gradient (0.1-1.2 M) in 7 M urea, pH 6.0. The column effluent was collected in fractions which were analyzed for contents of protein (absorbance 280 nm) hexuronic acid and sialic acid. Identical samples of the fractions were diluted with the incubation buffer for ELISA and the immunoreactivity against bone proteoglycans was analyzed by ELISA.

Immunofluorescence

Calf femoral epiphysis (age 4 months in utero) were carefully dissected free within 1 h after the death of the mother cow. Tissue specimens (about 2x2x4 mm) were embedded in carboxymethylcellulose (Tissue-Tek II, Miles lab., IL, USA), frozen on blocks of dry ice on a cryostat stage and sectioned at -30°C into 8 μ m sections. Each section was picked up on slides coated with gelatin. After being dried in the cryostat for about 1 h, the sections were fixed in absolute ethanol for 5 minutes and then left to dry at room temperature. Each section was incubated at 37°C for 30 minutes with 50 μ l of anti-bone proteoglycan antibodies diluted 1:10 with

PBS. Control sections were similarly incubated with diluted (1:10) preimmune serum from the rabbit. The sections were extensively washed with PBS for 1 h at 20°C. Swine anti-rabbit IgG conjugated with FITC (Dako, Denmark) and diluted to 1:25 with the buffer (50 µl) was added to each of the sections. After incubation at 37°C for 30 minutes, the sections were carefully washed at 20°C for 4 h with the buffer, distilled water and dried. Distribution of fluorescence was studied using a Leitz microscope and photographed at 20 x magnification with Kodac Ectachrome (400 ASA) film.

RESULTS AND DISCUSSION

Composition analysis

The bone proteoglycan has a high protein content of almost 40%, comparable to that of small proteoglycans from other sources (for ref. see Heinegård & Paulsson, 1984). Other similarities with such proteoglycans are relatively low contents of uronic acid and hexosamines (Table I). The amino acid composition is typical for small proteoglycans, showing high contents of aspartic acid/asparagine, glutamic acid/glutamine and leucine (Franzén & Heinegård, accompanying paper). No hydroxyproline could be demonstrated. The glucosamine to galactosamine ratio of the proteoglycan was 0.15. The presence of mannose, glucosamine and sialic acid can be attributed to oligosaccharides. Interestingly the small proteoglycan isolated from cartilage contains no sialic acid (Heinegård *et al.*, 1981a), showing that the molecules are not identical.

Characteristics of the carbohydrate side chains

Oligosaccharides and glycosaminoglycans were liberated by treatment of the proteoglycan with alkaline-borohydride. Chromatography on BioGel P10 (Fig. 1) revealed one major orcinol reactive component eluting in the void volume, which represents glycosaminoglycan chains. The oligosaccharides distributed in several components corresponding to the elution position of four to fourteen hyaluronic acid monosaccharide units; Five (I-V) fractions were pooled as indicated (Fig 1).

A sample of the glycosaminoglycans in the void volume fraction (I) was digested with chondroitinase ACII and chromatographed on Sephadex G50. This chromatogram showed, that the enzyme completely depolymerized the glycosaminoglycans to disaccharides showing that the side chains contain only chondroitin sulphate (data not shown).

The composition of the four oligosaccharide fractions (II-V) isolated after alkaline borohydride were determined (Table I). All fractions contained some galactosamine, possibly due to a contamination with low molecular weight chondroitin sulphate. The fractions (II,III) representing the longest oligosaccharides did not contain any hexosaminitol, indicating presence in these fractions of only oligosaccharides which are N-glycosidically linked to the protein core and therefore stable to the alkaline-borohydride treatment. These two oligosaccharide fractions differed with respect to composition. The fractions eluting retarded (IV,V) and therefore representing smaller oligosaccharides, contained galactosaminitol as well as glucosamine and hexose. Since these oligosaccharides contained galactosaminitol it is likely that they were O-glycosidically linked to the proteoglycan core protein. The sialic acid contents of the four fractions were quite similar. At present, we can not explain the high content of mannose in the two late eluting fractions (IV,V). Mannose would not normally be found in O-glycosidically linked oligosaccharides. The data, however, show that the bone proteoglycan contains chondroitin sulphate chains as well as both N- and O-glycosidically linked oligosaccharides.

Apparent molecular weight and sedimentation coefficient

The molecular weight of the bone proteoglycan is 74600 as determined by sedimentation-equilibrium centrifugation in 4 M guanidinium chloride (Fig. 2a). The same value was obtained both with measurements using the schlieren optics and with measurements of the absorbance at 280 nm, indicating that the contribution of free chondroitin sulphate chains, if any, was small. Determinations at three different centrifugation speeds (17000, 19000 and 21000 rev./min) gave identical values, indicating that the proteoglycan preparation was less polydisperse than often seen. Because of the great similarities in composition between the cartilage small proteoglycan and the bone proteoglycan, the partial specific volume, i.e. 0.59 ml/g, obtained for the cartilage proteoglycan was used for

calculating the molecular weight. Interestingly the molecular weight values were almost identical for the two proteoglycans. The $S_{20,w}$ value for the bone proteoglycan was 3.04 S determined by sedimentation-velocity centrifugation at 55000 rev./min in 4 M guanidinium chloride (Fig. 2b). The cartilage small proteoglycan had a similar $S_{20,w}$ value of 3.11 S, determined under identical conditions (Heinegård et al., 1981).

Apparent molecular weight of the proteoglycan core protein

The core protein prepared by digestion of the bone proteoglycan with chondroitinase ABC has an apparent molecular weight of 46000 determined by SDS polyacrylamide gel electrophoresis on 8% gels (Fig. 3). This value represents an overestimate, due to the shortcomings of the technique used. A better estimate of the molecular weight of the core protein is 30000, calculated from the protein content and the molecular weight of the intact proteoglycan. The electrophoresis, however, show that the core protein is monodisperse, i.e. one well defined component. Interestingly proteoglycans from other sources like cartilage (Heinegård et al., 1981), sclera (Cöster and Fransson, 1981), cervix (Uldbjerg et al., 1981), tendon (Vogel and Heinegård, unpublished) and aorta (Gardell and Heinegård, unpublished) have similar size and the core protein migrates on SDS polyacrylamide gel electrophoresis with an apparent molecular weight of 44-46000. It is possible that these proteoglycans represent a group of closely related molecules.

Molecular weight of the glycosaminoglycan chains

The molecular weight of the glycosaminoglycan side chains were determined by gel chromatography on Superose 6B of alkaline-borohydride treated bone proteoglycans (Fig. 4). M_n and M_w were calculated according to Wasteson (1971) and found to be 18600 and 33700 dalton, respectively. These values are similar to those obtained with the other similar proteoglycans discussed above. The average size of the glycosaminoglycan chains from this type of proteoglycan, however, are almost twice those of the large proteoglycans from bovine nasal cartilage (Heinegård et al., 1981).

Characteristics of the proteoglycan oligosaccharides

From the size determination of the intact bone proteoglycan and of fragments thereof, it is possible to postulate a tentative model of the molecule (Fig. 5). The molecule appear to contain a core protein with an

estimated molecular weight of 30000 and of one or two large glycosaminoglycan side chain of about 34-35000 dalton. The molecule in addition contains both N-linked and O-linked oligosaccharides.

Specificity of the antibodies

The purified bone proteoglycan was used for raising antibodies in rabbits. The specificity of the antibodies was tested with an inhibition ELISA. The macromolecules bound in the calcified matrix of bovine bone were extracted as previously described (Franzén and Heinegård, accompanying paper). The extract was chromatographed on a DEAE-cellulose ion exchange column eluted with a sodium acetate gradient, and the effluent fractions were analyzed for contents of protein, hexuronic acid and sialic acid (Fig. 6b). Three main polyanionic components were bound to the column and eluted well separated from each other at different concentrations of sodium acetate. The second component was identified as a bone sialoprotein, probably corresponding to that originally identified by Andrews et al. (1967). The bone proteoglycans, identified by their high hexuronic acid content were recovered in the third main peak. The immunoreactivity against bone proteoglycans was determined, by measuring the inhibition of each fraction in the ELISA (Fig. 6a). More than 98% of the bone proteoglycan immunoreactivity was recovered in the expected late eluting peak, showing the specificity of the assay. A very small portion (less than 2%) was found in the fractions containing material not bound to the column, probably representing a small proportion of bone proteoglycan not bound to the column. The data show that the antibodies did not cross-react (measured as inhibition in the ELISA) with any other component in the extract of bone.

Cross-reactivity of small proteoglycans

The small proteoglycans of bone and cartilage, showed similarities in the amino acid composition as well as in their hexosamine and hexuronic acid contents. Furthermore, the molecular weights of the two proteoglycans as well as the dimensions of the core proteins were quite similar. Therefore, the immunological relationship between the two molecules was studied (Fig. 7). The wells in the microtiter plate were coated with chondroitinase ABC digested bone proteoglycan. The inhibition in an assay for bone proteoglycan was tested with intact as well as with chondroitinase ABC digested small proteoglycans from bone and cartilage. All prepa-

rations reacted with the antibodies. Surprisingly the undigested proteoglycans showed a higher degree of inhibition than did those digested (Fig. 7). The small cartilage proteoglycan was a less efficient inhibitor than the bone proteoglycan indicating only partial cross-reactivity between the two small proteoglycans.

Immunofluorescence

The antibodies directed against the bone proteoglycans were used to localize these molecules within unfixed cryostat sections of fetal calf epiphysis. Indirect immunofluorescence with a double antibody technique, including anti-bone proteoglycan antibodies and anti-rabbit IgG labelled with FITC was used. Strong immunofluorescences with the specific antibody was only found in the primary spongiosa, while no immunofluorescence was detected among the hyperthrophic chondrocytes or in the early stages of the provisional calcification, Fig. 8. The normal cartilage of the resting zone showed weak immunofluorence, probably a result of some cross-reactivity between the small proteoglycans from cartilage and from bone. Substituting the specific antiserum with a preimmunization serum abolished all immunofluorescence (data not shown).

ACKNOWLEDGEMENTS

Grants were obtained from the Swedish medical research council (05668), Folksams yrkesskadors stiftelse, Kock's stiftelser, Österlunds stiftelse, Konung Gustaf V:s 80-års fond and the Faculties of Medicine and Odontology, University of Lund.

REFERENCES

- Andrews, A.T. deB, Herring, G.M. and Kent, P.W. (1967) *Biochem. J.* 104, 705-715
- Axelsson, I. and Heinegård, D. (1975) *Biochem. J.* 169, 517-530
- Carlson, D.M. (1968) *J. Biol. Chem.* 243, 616-626
- Cöster, L. and Fransson, L.-Å. (1981) *Biochem. J.* 193, 143-153
- Franzén, A. and Heinegård, D. Extraction and purification of proteoglycans from mature bovine bone. Accompanying paper
- Hascall, V.C. and Heinegård, D. (1974) *J. Biol. Chem.* 249, 4242-4249
- Heinegård, D. (1973) *Chem. Scr.* 4, 199-201
- Heinegård, D., Paulsson, M., Inerot, S. and Carlström, C. (1981) *Biochem. J.* 197, 355-366
- Heinegård, D. and Paulsson, M. (1984) in *Connective Tissue Biochemistry* (Piez, K. & Reddi, H., eds.), Elsevier-North Holland, New York, pp. 277-328.
- Inerot, S. and Heinegård, D. (1983) *Collagen Rel. Res. Vol. 3*, 245-262
- Jourdain, G.W., Dean, L. and Roseman, S. (1971) *J. Biol. Chem.* 246, 246, 430-435
- Kesler, R.B. (1967) *Anal. Chem.* 39, 1416 1422
- Lohmander, S., DeLuca, S., Nilsson, B., Hascall, V.C., Caputo, C., Kimura, J. and Heinegård, D. (1980) *J. Biol. Chem.* 255, 6084-6091
- Neville, D. (1971) *J. Biol. Chem.* 246, 6328-6334
- Stegeman, H. and Stalder, K. (1967) *Clin. Chim. Acta* 18, 267-273
- Uldbjerg, N., Malmström, A., Ekman, G., Sheehan, J., Ulmsten, U. and Wingerup, L. (1983) *Biochem. J.* 209, 497-503
- Wasteson, Å. (1971) *J. Chromatogr.* 59, 87-97
- Yphantis, D.A. (1964) *Biochemistry* 3, 297-317

Table I

Composition of the oligosaccharide fractions (II-V) prepared by chromatography on BioGel P-10 of alkaline-borohydride treated bone proteoglycans. The values are weight ratios ($\mu\text{g}/\text{mg}$ dry wt.) and number of residues per residue of glucosamine (within brackets).

	galactos- aminitol	glucos- amine	galactos- amine	galactos	mannose
II	-	0.89 (1)	2.47 (2.78)	1.03 (1.16)	0.98 (1.10)
III	-	2.38 (1)	1.82 (0.76)	1.26 (0.52)	2.81 (1.17)
IV	0.357 (0.18)	2.04 (1)	1.39 (0.68)	0.86 (0.42)	3.76 (1.85)
V	0.408 (1.41)	0.29 (1)	0.32 (1.11)	0.68 (2.35)	1.95 (6.75)

Table II

Distribution of hexosamines in the glycosaminoglycan and oligosaccharide fractions isolated from alkaline-borohydride treated bone proteoglycan by DEAE-cellulose chromatography (percent of proteoglycan dwt).

	glucosamine	galactosamine
glycosaminoglycans	0.2	11.6
oligosaccharides	1.6	0.5

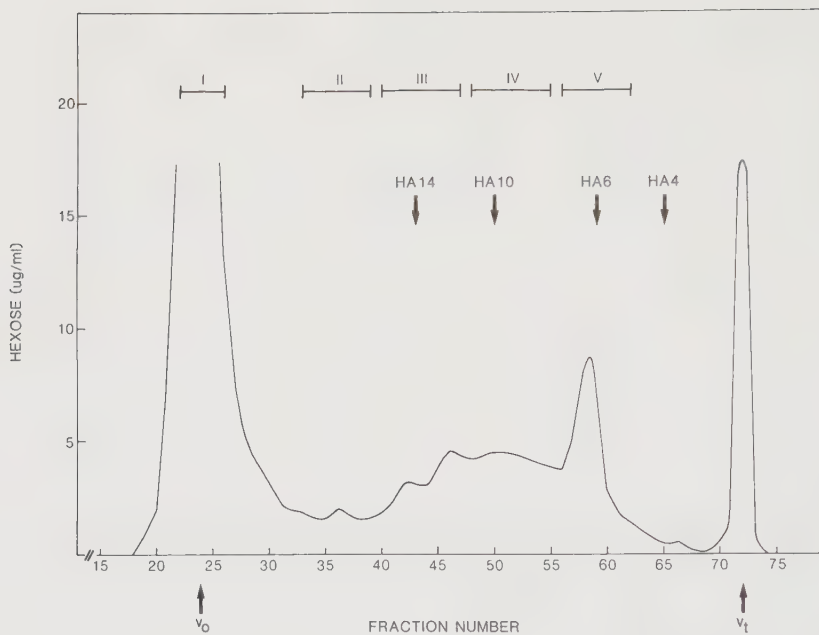


Figure 1: Gel chromatography of oligosaccharides. Bio-Gel P 10 chromatography (eluent 1 M pyridine-acetate, pH 7.0) of an alkaline-borohydride treated bone proteoglycan. — A₄₂₀ (orcinol reaction). Elution positions for hyaluronic acid oligomers are shown as reference. Further information is given in the text.

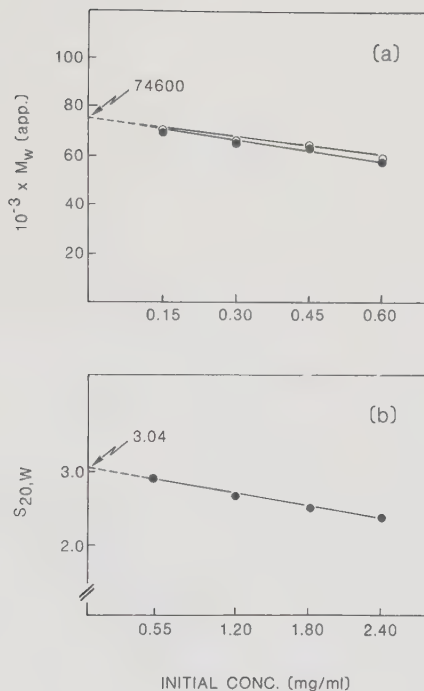


Figure 2: (a) Sedimentation characteristics of the bone proteoglycan. Molecular weight of the bone proteoglycan determined by sedimentation-equilibrium centrifugation in 4 M guanidinium chloride. The distribution of material was monitored using absorbance at 280 nm (○—○) and with Schlieren optics (●—●). For further information see Material and Methods. (b) Determination of $S^0_{20,w}$ of the bone proteoglycan using Schlieren optics. The sedimentation coefficients were calculated, corrected to 20°C and water and extrapolated to zero concentration.

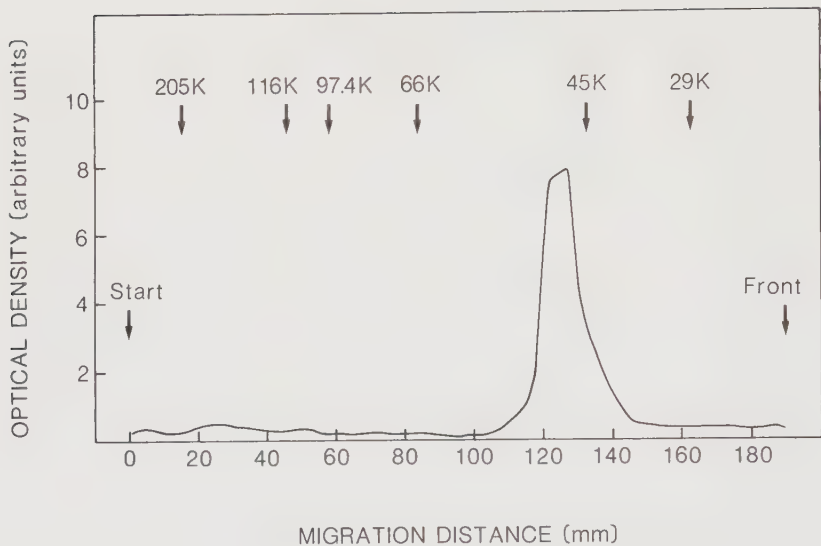


Figure 3: Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the bone proteoglycan after chondroitinase ABC digestion and reduction. The gel was scanned by using a soft laser scanner. The apparent molecular weight of the proteoglycan core protein was determined by comparing the migration distance with those of several reference proteins. Experimental details are given in the text.

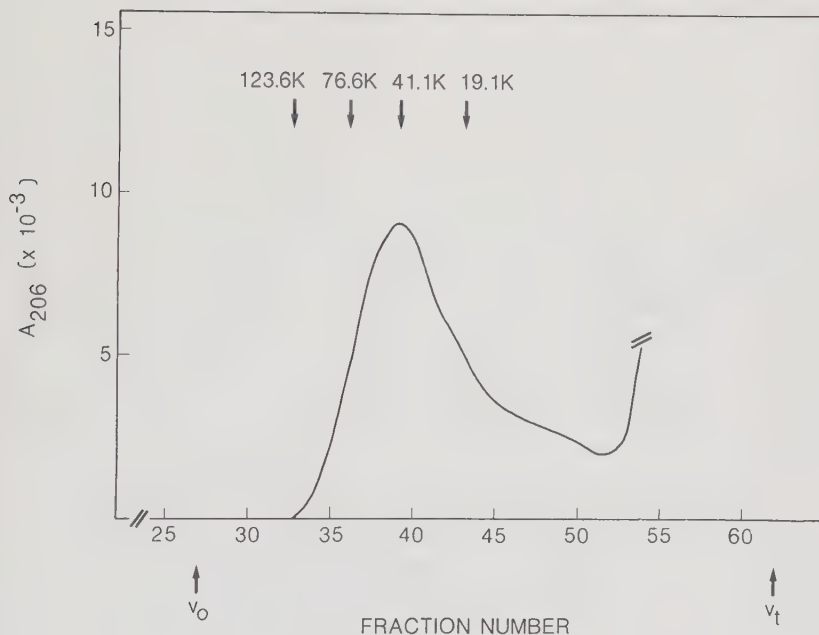


Figure 4: Determination of the apparent molecular weight of the glycosaminoglycan chains. The bone proteoglycan was treated with alkaline borohydride and the liberated chains were chromatographed on Sepharose 6B (eluent 0.25 M sodium sulphate, pH 7.0). The column was calibrated with chondroitin sulphate chains of known molecular weight. The column effluent was continuously monitored for absorbance at 206 nm using a UV-cord photometer. Experimental details are given in Materials and Methods.

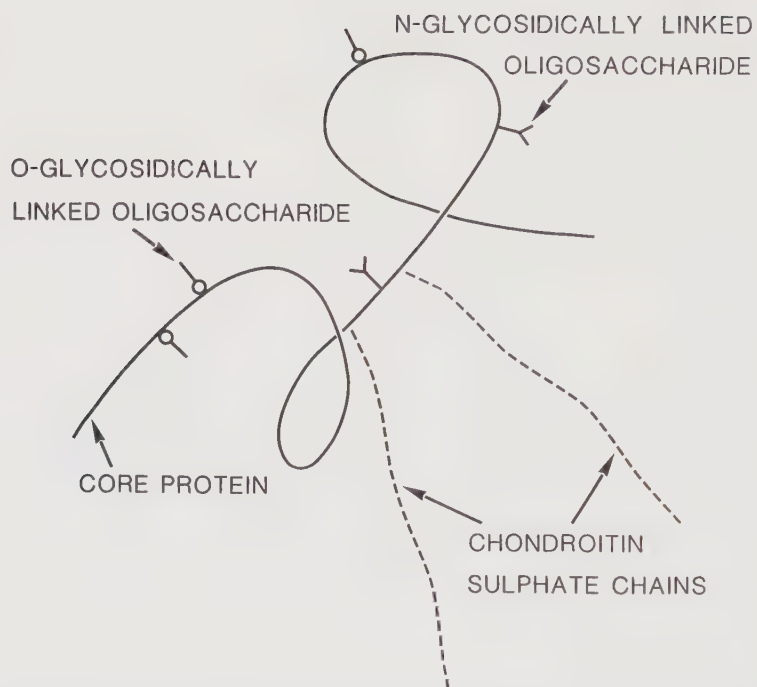


Figure 5: A tentative model of the isolated bone proteoglycan.

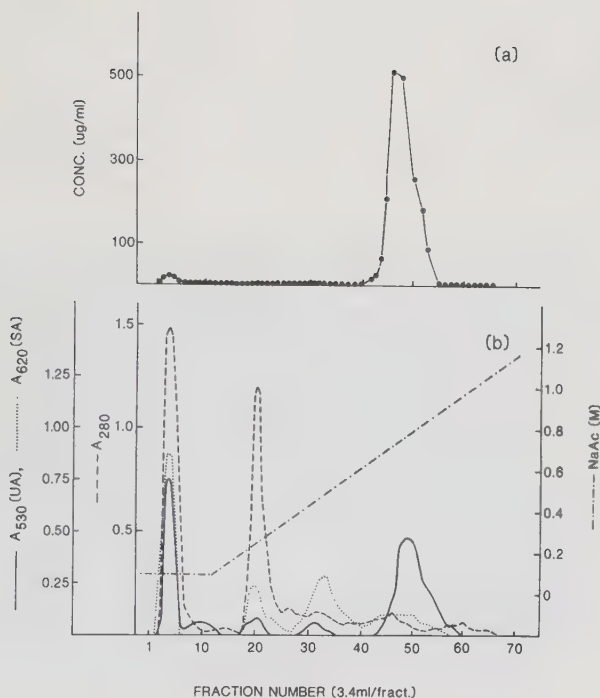


Figure 6: Specificity of ELISA of bone proteoglycan. DEAE-cellulose (DE-52) chromatography (eluent 7 M urea pH 6.0) of an extract with guanidinium chloride, EDTA of the calcified matrix of bovine bone. The column was eluted with a gradient of sodium acetate (—·—·—).

(a) Immunoreactivity against bone proteoglycans measured as inhibition in the ELISA.

(b) Protein at 280 nm (—·—·—), uronic acid by the carbozole reaction at 530 nm (——) and sialic acid (.....).

Experimental details are given in Material and Methods.

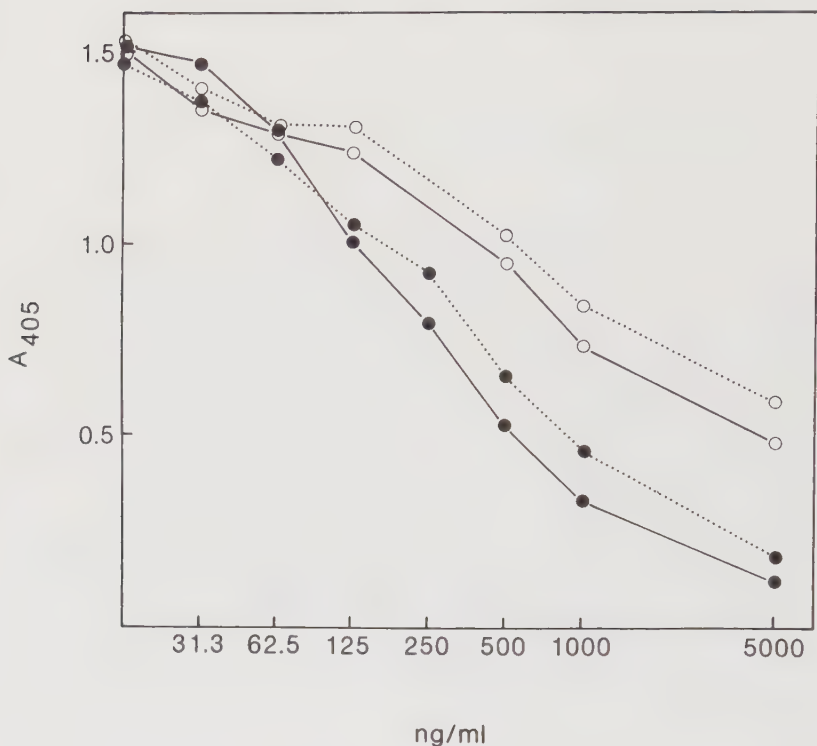


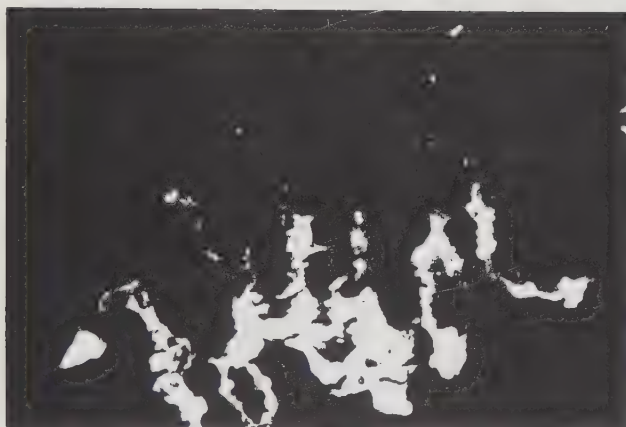
Figure 7: Immunological cross-reactivity of bone proteoglycan and cartilage small proteoglycan. ELISA for bone proteoglycans and inhibition with fractions indicated.

Bone proteoglycan (●—●)

Bone proteoglycans digested with chondroitinase ABC (●.....●)

Cartilage, small proteoglycan (○—○)

Cartilage, small proteoglycans digested with chondroitinase ABC (○.....○)



Hypertrophic
chondrocytes

Provisional
calcification

Primary
spongiosa

Figure 8: Localization of bone proteoglycan by immunofluorescens. Frozen, unfixed sections of fetal calf epiphysis were stained for bone proteoglycans by using double antibody immunofluorescence. (120X)

Zonal Rate Centrifugation of Proteoglycans in Sucrose Gradients

AHNDRERS FRANZÉN, SVEN BJÖRNSSON, AND DICK HEINEGÅRD

Department of Physiological Chemistry, University of Lund, P.O. Box 750, S-220 07 Lund 7, Sweden

Received June 4, 1981

Sensitive detection of proteoglycan and protein in concentrated sucrose solutions is obtained by measuring the absorbance at 206 nm. This method for detection has been used to study conditions affecting zonal rate sedimentation of proteoglycans and proteins in sucrose gradients. The ionic environment in the gradients markedly affects the sedimentation rate of the polyanionic proteoglycans and fragments thereof, while proteins sediment at an approximately constant rate irrespective of the ionic strength. The procedure can be used to separate proteoglycan aggregates from monomers and to determine size distributions of the compounds. In separate experiments, intact and fragmented proteoglycans are separated from proteins.

Proteoglycans are large macromolecules containing a central protein core with a number of negatively charged polysaccharide side chains radiating out in a comblike fashion (1-3). Their large hydrodynamic size and their large number of negatively charged groups hamper their fractionation according to size by gel chromatography, or according to charge by ion exchange chromatography. To date, however, some data on size distributions have been gathered by analytical ultracentrifugation (1) and by gel chromatography on low percentage agarose gels (4). Some proteoglycans, in particular from cartilage, form very large complexes. Several proteoglycan monomers can bind noncovalently to one hyaluronic acid molecule (5,6). The proteoglycan aggregate formed has a molecular weight of 100×10^6 or more. Most fractionation techniques do not allow fractionation of such large particles. The only tool suited for studies of the size distribution of proteoglycan aggregates has been the analytical ultracentrifuge.

Because of the problems associated with fractionating proteoglycans, much interest has been devoted to alternative techniques. An elegant micromethodology to determine *S* values of aggregates and monomers has

been developed by Pita (7) using sedimentation in CsCl gradients. The rather tedious procedure to prepare and analyze these gradients, however, make them unsuitable for analyses of large numbers of samples. Radioactively labeled proteoglycans from chondrocyte or limb bud cell cultures have been fractionated by zonal rate centrifugation in gradients of sucrose or glycerol to yield 2-3 populations of proteoglycan monomers (8-10). In other studies purified proteoglycans have been fractionated into aggregate and monomer by zonal rate centrifugation in NaCl gradients (11; Cleland, R. L., personal communication). The implications of these investigations are limited as the sedimentation behavior of monomers and aggregates in these gradients has not been characterized.

The advantages of zonal rate centrifugation are several. It is possible to fractionate even the largest molecular size material. As compared to chromatographic procedures, the absence of a matrix minimizes losses due to unspecific adsorption, a feature essential for analysis of small amounts of sample.

The aim of the present investigation was to develop a sensitive procedure to fractionate and detect proteoglycans and other mac-

romolecules in sucrose gradients. Sucrose was the gradient-forming medium of choice, as the ionic environment can be varied within wide limits. Alternative gradient media could be glycerol, methrizamide, or salts such as cesium chloride and cesium sulfate.

MATERIALS AND METHODS

Bovine articular, nasal, and tracheal cartilages were obtained from the abattoir within minutes after the death of the animal. Cartilage was dissected free from surrounding tissue, sliced into small pieces and stored frozen at -60°C until extraction.

Extraction with 4 M guanidinium chloride and subsequent preparation of proteoglycan aggregate (A1) and monomer (A1-D1) fractions by CsCl density gradient centrifugation was done as described elsewhere (12,13).

A sample (40 mg) of bovine nasal proteoglycan monomers were subfractionated according to size by chromatography on a Sepharose CL2B column (2.5×145 cm) eluted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.8. The effluent material was pooled to yield 8 subfractions.

Chondroitin sulfate peptides (clusters) were prepared by trypsin-chymotrypsin digestion of proteoglycans, separated by chromatography on Sepharose 6B, and then extensively characterized as described elsewhere (14). Chondroitin sulfate chains were prepared from bovine nasal cartilage by papain digestion and precipitation with cetylpyridinium chloride (15).

Samples to be centrifuged were dissolved in the appropriate buffer (usually 0.15 M sodium chloride, 5 mM sodium phosphate, pH 7.0) at the required concentration and slowly stirred in the cold for 12 h.

Chemical methods. Protein content of fractions was determined using the Lowry procedure (16) and hexosamine contents were determined with a modification of the Elson-Morgan procedure (15). All chemicals used were of analytical grade. Sepharose and Sephadex gels were purchased

from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of sucrose gradients. Solutions of 10, 30, and 50% sucrose containing the required salt and buffer were prepared. Usually 0.50 M sodium chloride, 5 mM sodium phosphate, pH 7.0, was used. All solutions were filtered through a Millipore type AA filter ($0.80 \mu\text{m}$) to remove particles. Monitoring the absorbance of the gradients at 206 nm required the background absorbance of the solutions to be adjusted to the same value. The absorbance at 206 nm (at 0.05 or 0.02 absorbance unit, full-scale deflection) was recorded while the most concentrated solution was continuously pumped through a Uvicord S (LKB, Sweden) flow-through photometer. The absorbance of the less concentrated solution could then be adjusted to the same value by addition of a few drops of an aqueous solution of a compound with a high absorbance at 206 nm, while the solution was recirculated through the Uvicord. Usually 0.2% (w/v) sodium azide or 8 M urea was used. The use of a peristaltic pump giving negligible pulses was found important for achieving stable baseline and a high sensitivity. In our experiments an LKB Multiperpex pump was used. The gradients were prepared by using two peristaltic pumps and a mixing chamber with a rotating magnetic bar. The sample solution was layered on top of the gradient by using a Hamilton gastight syringe. The gradients usually had a volume of 3.6 ml with $100 \mu\text{l}$ of sample in $\text{MSE } 6 \times 4.2$ polycarbonate tubes (70 mm length). The MSE Centriscan centrifuge was used with a 6×4.2 -ml titanium swing-out rotor. Centrifugation was done at 50,000 rpm (240,000g; r_{av} 8.802 cm) and a temperature of 20°C . The centrifuge was operated with the brake on during deceleration. In other experiments using conventional preparative ultracentrifuges it has been found better not to use the brake.

After centrifugation the tubes were emptied. A capillary was carefully pushed through the gradient to rest at the bottom

of the tube. The capillary was connected by Teflon tubing to the Uvicord S, which in turn was connected by Teflon tubing to a Multiperpex pump fitted with the 1-mm-i.d. pump tubing. The pump was connected to an LKB Redirac fraction collector. It is essential that the entire system is filled with the most concentrated sucrose solution before use, since the gradient has to be pumped from the top of the Uvicord flow cell rather than from the bottom as is usually recommended. It is also important to remove any air bubbles in the system before the gradient is emptied. The particular pump used is equipped with a stepmotor which can regulate the fraction collector to change after a defined volume has been pumped. Fractions were collected using this feature, mainly to indicate the volume of the gradient emptied at each moment. Usually a setting of the Uvicord of 0.2 A, full scale deflection, was used.

RESULTS AND DISCUSSION

Analysis of the Sucrose Gradients

In an initial experiment, nasal cartilage proteoglycan A1 fraction was fractionated to yield aggregate and monomer. After centrifugation for 36 min in a 10–50% sucrose gradient, the tube was emptied and the effluent was continuously monitored for absorbance at 206 nm using the Uvicord S. The fractions collected were extensively dialyzed against water and freeze-dried. They were then redissolved in distilled water and analyzed for contents of protein. Aliquots were withdrawn, hydrolyzed, and hexosamine contents were determined. The profiles for uv absorbance of protein and hexosamine closely follow each other (Fig. 1) indicating that any of these measures can be used equally well to monitor for proteoglycan distribution. Indeed, at 206 nm proteins have a very strong absorbance. At this wavelength the *N*-acetamido and carboxyl groups of the glycosaminoglycans will provide much

higher absorbance than simple sugars such as sucrose. In a separate experiment, a protein (bovine serum albumin), on a weight basis, was found to give six times the absorbance of protein-free chondroitin sulfate. The absorbance value recorded for a cartilage proteoglycan, then, is approximately half due to protein and half due to glycosaminoglycans. The profiles in Fig. 1 show a very broad, faster-sedimenting peak containing the proteoglycan aggregates, and a slower-sedimenting peak remaining near the top of the tube containing the proteoglycan monomers. The aggregates appear polydisperse and contain some extremely fast sedimenting material. The proportion of aggregate to monomer was about 85:15, which is in accordance with previous data (6).

Supporting data have been obtained by centrifugation under identical conditions of proteoglycan monomers with and without added hyaluronic acid. The hyaluronic acid-proteoglycan aggregate was shown to be stable to the conditions of centrifugation and to sediment like the link stable aggregates studied in the present paper, whereas monomers sediment slowly and show as a sharp peak near the top of the gradient at such short centrifugation times (17).

Characteristics of the Sedimentation

Dependence on the concentration of sucrose. The sedimentation distance of proteoglycan monomers as a function of time was determined for 10 to 30% and 10 to 50% (w/v) linear sucrose gradients (Fig. 2A). As expected, the sedimentation was slower in 10 to 50% sucrose gradients and was linear only for 2 to 3 h of centrifugation, corresponding to 30–35% of the tube length. At this time the sedimentation rate decreased. In contrast, sedimentation in 10 to 30% sucrose gradients was linear with time for at least 5 h, corresponding to about 80% of the tube length. The 10–30% sucrose gradients appear isokinetic and should be preferred for most applications. To prevent the fastest-

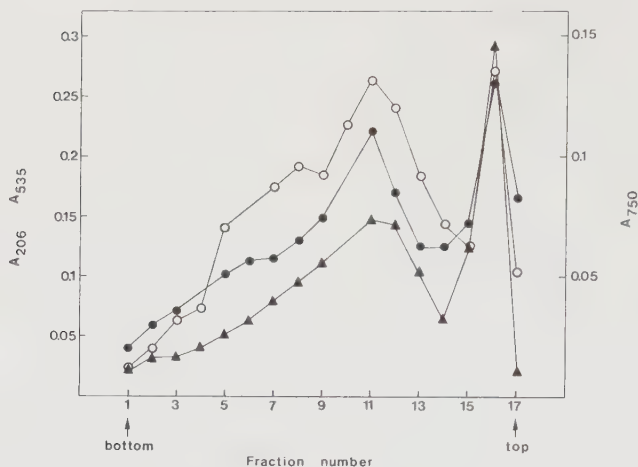


FIG. 1. Zonal rate sedimentation of nasal cartilage A1 fraction in 10 to 50% sucrose. Analysis for absorbance at 206 nm (▲), hexosamine (○), and protein (●).

sedimenting proteoglycan aggregates from accumulating at the bottom of the tube, the 10–50% sucrose gradients were used when aggregate-containing samples were centrifuged.

Dependence on the concentration of proteoglycan. The concentration dependence of sedimentation of proteoglycan aggregates and monomers in proteoglycan A1 fraction was studied (Fig. 2B). Centrifugation was for either 36 or 60 min in 10 to 50% sucrose gradients. By using a full-scale deflection of 0.05 A unit of the Uvicord it was possible to analyse the A1 fraction at 0.19 mg/ml (19 μ g/sample). Notably, the concentration dependence was very small for the sedimentation of both aggregate and monomer at the wide range of concentrations monitored (Fig. 2B). Therefore, the procedure should be useful also for studying samples for which the exact concentration of proteoglycan is not known.

Dependence on ionic environment. The effect of salt concentration and type of salt on the sedimentation properties of protein,

proteoglycan, and fragments thereof was studied. Albumin was chosen as a model protein. Nasal cartilage proteoglycan monomers (A1–D1) were used for reference. Proteoglycan trypsin and trypsin–chymotrypsin fragments, which represent chondroitin sulfate peptide clusters (15) were centrifuged as well as hyaluronic acid and isolated chondroitin sulfate chains. Consequently, heavily charged (polyanionic) sulfated macromolecules of large, intermediate and small size (proteoglycans, chondroitin sulfate, peptides and chains, respectively), less-charged large macromolecules (hyaluronic acid) containing no sulfate groups, and little-charged molecules (albumin) were sedimented in the sucrose gradients. Three types of centrifugation conditions with regard to ionic strength were tried. Centrifugation was done at low ionic strength (0.005 M sodium phosphate) and in two different salt environments at intermediate ionic strength (0.5 M sodium chloride and 0.5 M CsCl, respectively, both in 0.005 M sodium phosphate). In each case 300 μ g of sample

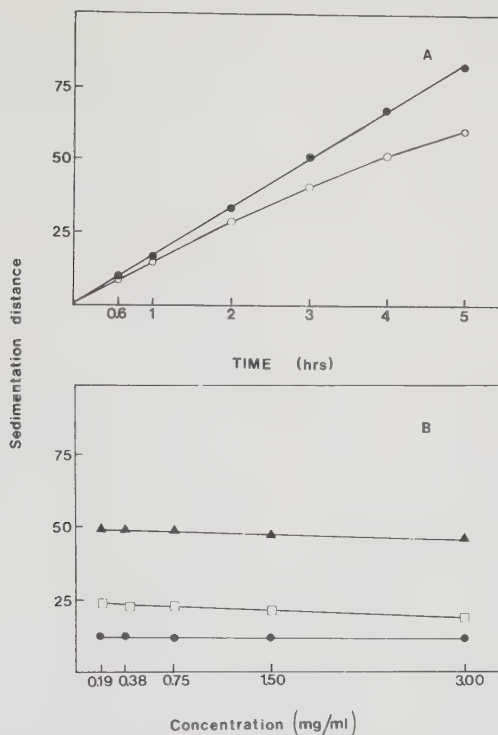


FIG. 2. (A) Sedimentation dependence on duration of centrifugation. 10-30% sucrose (●), 10-50% sucrose (○). Sedimentation expressed as distance sedimented over distance meniscus to bottom of tube. (B) Concentration dependence of sedimentation rate in 10 to 50% sucrose gradients of aggregate (▲) and monomers (●) centrifuged for 36 min and for monomers (□) centrifuged for 60 min.

was centrifuged. Centrifugation times had to be varied due to the marked influence of the salt on the sedimentation properties of the polyanions.

In all conditions proteoglycans sedimented faster than any of the fragments thereof or albumin (Fig. 3). Trypsin fragments sedimented faster than the smaller trypsin-chymotrypsin fragments and chondroitin sulfate chains as expected (Fig. 3). The relative sedimentation rate of proteoglycan, chondroitin sulfate peptides, chondroitin sulfate chains, and hyaluronic acid

was similar in all conditions of centrifugation. The absolute sedimentation, however, was fastest in CsCl, intermediate in sodium chloride, and markedly slow at low salt concentration. In contrast the sedimentation rate of albumin was rather constant in the different ionic environments (Fig. 3). The experiments show that by altering the ionic environment in the gradient it is possible to change the sedimentation rate of polyanionic proteoglycans and chondroitin sulfate peptides. It is important to note that polyanionic macromolecules (in contrast to proteins) are

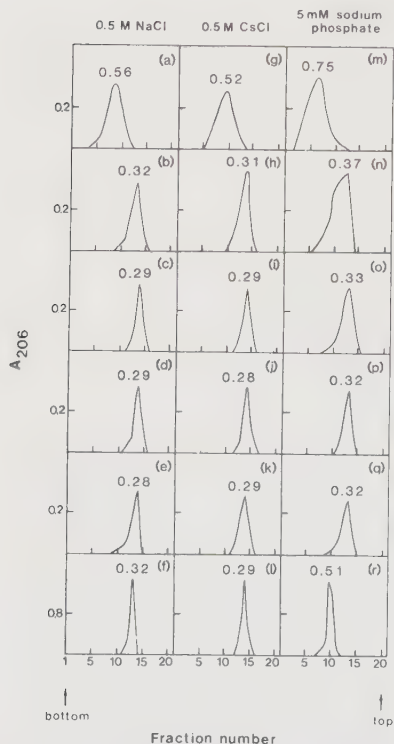


FIG. 3. (a, g, m) Sedimentation of proteoglycan monomers; (b, h, n) chondroitin sulfate (CS) peptides liberated from proteoglycan monomers by trypsin digestion; (c, i, o) chondroitin sulfate peptides liberated from proteoglycan monomers by sequential digestion with trypsin-chymotrypsin; (d, j, p) chondroitin sulfate chains isolated from proteoglycan monomers; (e, k, q) high-molecular-weight hyaluronic acid (Healon) and (f, l, r) albumin in various ionic conditions. Centrifugation in 10 to 50% sucrose containing 0.5 M sodium chloride, 0.005 M sodium phosphate, pH 7 (a-f), 0.5 M cesium chloride, 0.005 M sodium phosphate, pH 7 (g-l), and in 0.005 M sodium phosphate, pH 7 (m-r). Samples were dissolved in 0.15 M sodium chloride. 0.005 M sodium phosphate, pH 7 (a-f), 0.15 M cesium chloride, 0.005 M sodium phosphate, pH 7 (g-l), and 0.005 M sodium phosphate, pH 7 (m-r). Centrifugation in 0.5 M NaCl for 3 h (a-f), in 0.5 M CsCl for 1.5 h (g-l), and in 0.005 M sodium phosphate for 10 h (m-r). The numbers express the distance sedimented over distance meniscus to bottom of tube.

expanded at low ionic strength and therefore sediment relatively slower. At higher ionic strength the polyanionic macromolecules are more compact and therefore sediment faster. In gradients containing 0.5 M CsCl, good separation of intact proteoglycan monomer and albumin was achieved. By using divalent ions or compounds interacting more strongly with the sulfate groups or with particular populations of proteoglycans, it is also possible to improve the separation of proteoglycan classes containing different glycosaminoglycan side chains. Indeed, in separate experiments it was shown that calcium ions may affect the sedimentation properties of proteoglycans (data not shown).

Size of proteoglycan monomer. The fractionation of proteoglycans according to size was studied using both 10–50 and 10–30% sucrose gradients. A sample of proteoglycan monomer was subfractionated according to size by gel chromatography on Sepharose CL-2B (Fig. 4). Three of the subfractions obtained were centrifuged in the sucrose gradients for 3 h (at $240,000g_{av}$) (Fig. 4). The more retarded on the Sepharose column, the lower was the sedimentation rate of the sample, indicating that the separation system indeed does fractionate according to size, which in the case of proteoglycans is related to molecular weight.

Size of aggregate. Bovine tracheal cartilage proteoglycan aggregates have a lower *S* value and are smaller compared with those from bovine nasal cartilage (6). They were found to differ accordingly with regard to their sedimentation behavior in the sucrose gradients (Fig. 5). The aggregates from bovine articular cartilage sedimented even slower (Fig. 5). The monomers from tracheal and nasal cartilage, however, sedimented with approximately the same rate (Fig. 5), corroborating previous data (6). The results obtained show that zonal rate sedimentation in sucrose gradients can be used to monitor size of aggregates as well as ratio of proteoglycan aggregate to monomer.

GENERAL DISCUSSION

Zonal rate centrifugation in sucrose gradients has been shown to be an extremely useful tool in fractionating proteoglycans. The important advantages include the absence of matrix, no upper limit on size determinations, ease of running multiple samples, speed of assay and a much improved

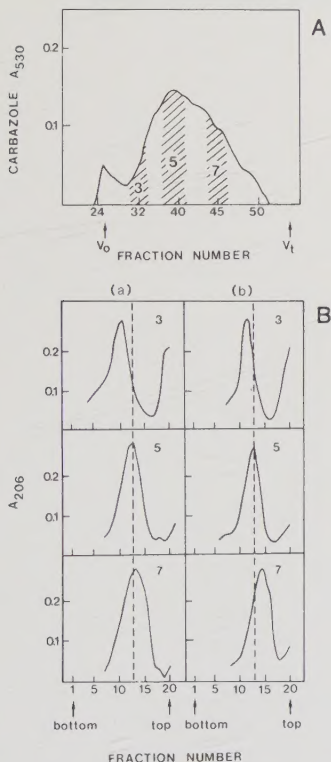


FIG. 4. (A) Fractionation of proteoglycan monomers on a Sepharose CL-2B column eluted with 4 M guanidinium chloride. The fractions indicated were pooled, dialyzed against sodium acetate and distilled water, freeze-dried, and used for sucrose zonal rate centrifugation. (B) Zonal rate sedimentation in (a) 10 to 30% and (b) 10 to 50% sucrose gradients of the samples recovered from the Sepharose CL-2B column in (A).

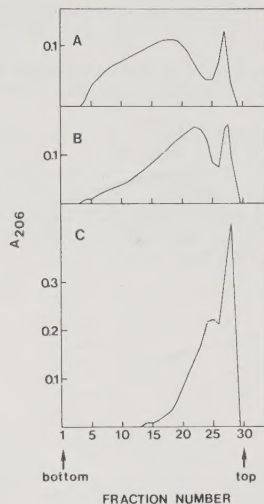


FIG. 5. Zonal rate sedimentation for 36 min in 10 to 50% sucrose gradients of proteoglycan A1 fractions from (A) bovine nasal cartilage, (B) bovine tracheal cartilage, and (C) bovine articular cartilage.

sensitivity. As little as 20 μ g of proteoglycan suffices for studying proportion of aggregate and monomer and their respective size distribution.

ACKNOWLEDGMENTS

Grants were obtained from the Swedish Medical Research Council (13P-5739 and 13X-5668), Konung Gustaf V:s 80-års fond, Kocks Stiftelser, and the Medical Faculty, University of Lund.

REFERENCES

1. Hascall, V. C., and Sajdera, S. W. (1970) *J. Biol. Chem.* **245**, 4920-4930.
2. Rosenberg, L., Hellman, W., and Kleinschmidt, A. K. (1975) *J. Biol. Chem.* **250**, 1877-1883.
3. Thyberg, J., Lohmander, S., and Heinegård, D. (1975) *Biochem. J.* **151**, 157-166.
4. Heinegård, D. (1977) *J. Biol. Chem.* **252**, 1980-1989.
5. Hardingham, T., and Muir, H. (1972) *Biochim. Biophys. Acta* **279**, 401-405.
6. Hascall, V. C., and Heinegård, D. (1974a) *J. Biol. Chem.* **249**, 4232-4241.

7. Pita, J. C., Muller, F. J., Morales, S. M., and Alarcon, E. J. (1979) *J. Biol. Chem.* **254**, 10313-10320.
8. Kato, Y., Kimata, K., Ito, K., Karasawa, K., and Suzuki, S. (1978) *J. Biol. Chem.* **253**, 2784-2789.
9. Vasan, N. S., and Lash, J. W. (1979) *J. Embryol. Exp. Morphol.* **49**, 47-59.
10. Karasawa, K., Kimata, K., Ito, K., Kato, Y. and Suzuki, S. (1979) *Dev. Biol.* **70**, 287-305.
11. Hoffman, P. (1979) *J. Biol. Chem.* **254**, 1854-1860.
12. Heinegård, D., and Axelsson, I. (1977) *J. Biol. Chem.* **252**, 1971-1979.
13. Heinegård, D., and Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 927-934.
14. Heinegård, D., and Hascall, V. C. (1974) *Arch. Biochem. Biophys.* **165**, 427-441.
15. Antonopoulos, C. A., Gardell, S., Szirmai, J., deTyssonsk, E. (1964) *Biochim. Biophys. Acta* **83**, 1-19.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
17. Franzén, A., Björnsson, S., and Heinegård, D. (1981) *Biochem. J.* **197**, 669-674.

